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Regulation of S-adenosylmethionine synthetase genes in amoebae by bacterial endosymbionts

Taeck-Joong Jeon

To the Graduate Council:

I am submitting herewith a dissertation written by Taeck-Joong Jeon entitled "Regulation of S-adenosylmethionine synthetase genes in amoebae by bacterial endosymbionts." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

Kwang W. Jeon, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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REGULATION OF S-ADENOSYLMETHIONINE SYNTHETASE GENES IN AMOEBAE BY BACTERIAL ENDOSYMBIONTS

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Taeck-Joong Jeon

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DEDICATION

This dissertation is dedicated to my parents

Mr. Byung-In Jeon and Mrs. Joong-Ok Nam

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ABSTRACT

The xD strain of *Amoeba proteus* that arose from the D strain by spontaneous infection of X-bacteria is now dependent on X-bacteria for survival. The S-adenosylmethionine synthetase (SAMS) is the enzyme that catalyzes the synthesis of S-adenosylmethionine (SAM), a major methyl donor in cells. As a result of harboring obligatory bacterial endosymbionts, xD amoebae do not transcribe the *sams* gene and no longer produce their own SAMS. When symbiont-free D amoebae are infected with symbionts (X-bacteria), the amount of amoeba SAMS decreases to a negligible level within four weeks, but about 47% of the SAMS activity, which apparently comes from another source, is still detected. Therefore, it was postulated that X-bacteria suppressed the expression of amoeba's *sams* and in turn provided the enzyme of SAM for their hosts, forcing host amoebae to become dependent on symbionts themselves.

As a part of my work to elucidate the mechanism for the development of mutual dependence between symbionts and hosts, first the *sams* genes of amoebae and X-bacteria were cloned and characterized (PART II). The previously reported *sams* gene of amoebae turned out to be an isoform, so now we call the gene *sams1*. The open reading frame of the amoeba's *sams1* gene has 1,281 nucleotides, encoding SAMS of 426 amino acids with a mass of 48 kDa and pI of 6.5. The 5'-flanking region of amoeba *sams1* contains consensus-binding sites for several transcription factors that are related to the regulation of *sams* genes in *E. coli* and yeast. The open reading frame of X-bacteria *sams* is 1,146 nucleotides long, encoding SAMS of 381 amino acids with a mass of 41 kDa and pI of 6.0. The X-bacteria SAMS has 45% sequence identity with that of *A. proteus*.

Next I tested if symbionts supplied SAMS or SAM to host xD amoebae by localizing X-bacteria SAMS in xD amoebae and measuring SAMS activities in D and xD amoebae (PART III). The results show that amoebae have a second *sams* (*sams2*) gene, encoding 390 amino acids and that the SAMS activity found in xD amoebae comes from SAMS2 not from the endosymbionts. Interestingly, the expression of two amoeba *sams* genes was switched from *sams1* to *sams2* by infection with X-bacteria, raising a possibility that the switch in the expression of *sams* genes by bacteria plays a role in the

development of symbiosis and the host-pathogen interactions. This is the first report showing the switch in the expression of host *sams* genes by infecting bacteria.

In PART IV, the analysis for DNA adenine methylation of *sams1* shows that the *sams1* gene is methylated at an internal adenine residue of GATC site in xD amoebae whereas no methylation occurs in D amoebae. This result implies that the modification might be the reason for the suppression of *sams1* in xD amoebae. The methylation of cytosine residue in CpG sites is the most common modification in eukaryotes. However, it appears that the methylation of cytosine residues is not responsible for the inactivation of *sams1* in xD amoebae. DNA analysis shows that adenine residues in X-bacteria *sams* are also methylated, indicating *Legionella*-like X-bacteria belong to Dam methylase-positive strains. In addition, it appears that SAM and methionine act as a negative regulator for the expression of *sams1* whereas the expression of *sams2* is not affected in amoebae. After the removal of X-bacteria from xD amoebae, the *sams1* gene was reactivated and *sams2* was down-regulated. The results are reversed in the expression of *sams* genes when amoebae are infected with X-bacteria. This is the first report to show that a specific eukaryotic gene is modified by DNA adenine methylation.

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PART I

GENERAL INTRODUCTION AND OVERVIEW

CHAPTER 1
AMOEBA/X-BACTERIA SYMBIOSIS

A stable endosymbiosis is established by a series of complex interactions between prospective hosts and symbionts, including surface recognition, entry of one partner into the other, avoidance of destruction by the host partner, and sustained multiplication of both partners. Much has been studied so far about each step using several kinds of systems, but the elucidation of mechanisms for endosymbiosis is rarely possible.

Amoeba/X-bacteria symbiosis was established by spontaneous infection of a strain of *Amoeba proteus* with a large number of gram-negative and rod-shaped bacteria (X-bacteria) and then development of a stable symbiosis within a short period of time under laboratory conditions in 1966 (Jeon and Lorch 1967). When the bacteria first infected amoebae, they were virulent and killed whole cultures of newly infected amoebae. While most of the original nonsymbiotic strain (D amoebae) infected by X-bacteria died, a few of them survived and X-bacteria gradually lost their virulence. The number of symbionts, X-bacteria, in each symbiont-bearing amoeba (xD amoebae) was stabilized at about 42,000, and the host xD amoebae grew normally under standard culture conditions (Ahn and Jeon 1979). However, as a result of symbiosis with X-bacteria, the host xD amoebae showed some physiological changes such as temperature sensitive and overfeeding or starvation sensitive, and the hosts became dependent on their symbionts for survival within 18months (200 cell generations) (Jeon and Ahn 1978). This amoeba/X-bacteria symbiosis is different from other symbiotic systems in that the history of the establishment of symbiosis is known, symbionts are essential for the host's survival, and cellular character changes including the host's dependence can be experimentally reproduced under laboratory conditions. This amoeba/X-bacteria symbiosis offers extraordinary opportunities for studying the establishment of new symbiotic associations and host-symbiont interactions (Jeon 1995).

Symbiont-induced genetic change in host xD amoebae. Genetic alteration of the host's gene expression in the amoeba/X-bacteria symbiosis was postulated based on several

observations. Supporting data include; infected xD amoebae cannot grow if their symbionts are removed (Jeon and Ahn 1978). Nuclei of newly infected D amoebae change within a few weeks and are not compatible with the cytoplasm of D amoebae as a result of harboring bacteria. The nucleus of a xD amoeba is no longer the same as that of a D amoeba (Lorch and Jeon 1981; Lorch and Jeon 1982). For investigating the genetic change in xD amoebae, the protein composition of D and xD amoebae was analyzed by two-dimensional polyacrylamide gel electrophoresis (Ahn and Jeon 1983). In this analysis, it was found that 45-kDa protein was present only in D amoebae and disappeared from the newly infected xD amoebae within 50 days after infection (Ahn and Choi 1985; Ahn and Jeon 1983). Finally, the D-specific 45-kDa protein turned out to be S-adenosylmethionine synthetase (SAMS) by screening an amoeba-cDNA library with monoclonal antibodies (mAb) against the D-specific 45-kDa protein (Choi et al. 1997). It has been reported that symbiont-bearing xD amoebae do not transcribe the *sams* gene and no longer produce their own SAMS as a result of harboring X-bacteria. Meanwhile, xD amoebae still show about half the level of SAMS activity found in symbiont-free D amoebae (Choi et al. 1997).

CHAPTER 2
S-ADENOSYLMETHIONINE SYNTHETASE (SAMS)

SAMS catalyses the formation of S-adenosylmethionine (SAM) using methionine and ATP (L-Methionine + ATP → SAM +PP_i +P_i). SAM is involved in a great number of cellular reactions (Mato et al. 1997). First, SAM is known to be the principal methyl donor in methylation of cytosine or adenine of DNA, rRNA, tRNA, and various proteins. Second, the carboxyaminopropyl group can be used for the synthesis of modified nucleotides in rRNA, or, after the decarboxylation of SAM, the amino-propyl group is used for polyamine synthesis (Thomas and Surdin-Kerjan 1997). SAM is also used as an amino-group donor in the synthesis of pelargonic acid, a precursor of biotin (Stoner and Eisenberg 1975) and serves as ribosyl-group donor in the synthesis of queuine, a modified base of tRNA of prokaryotes and eukaryotes (Slany et al. 1993). In plants, SAM is cleaved to methylthioadenosine and aminocyclopropane carboxylic acid, a precursor of ethylene, the fruit-ripening hormone (Theologis 1992). SAM is also known as an effector in the negative regulation of sulfur amino acid metabolism in yeast (Jacquemin-Faure et al. 1994) and in the regulation of methionine metabolism in mammals (Mato et al. 1997).

***S-adenosylmethionine synthetase* (*sams*) genes.** The *sams* genes have been cloned from bacteria, fungi, plants and animals and shown to be evolutionarily well conserved (Thomas and Surdin-Kerjan 1997). The presence of two different SAMS in *S. cerevisiae* was recognized by DEAE-cellulose chromatography, and the two different *sams* genes (*sam1* and *sam2*) were cloned (Thomas et al. 1988). The products of the *sam1* and *sam2* genes are 92% identical with each other and closely resemble *E. coli* SAMS with 52% of identity. Two SAMS-encoding genes have been reported in *E. coli* (Satischandran et al. 1993) and in *Arabidopsis thaliana* (Peleman et al. 1989). In mammals, there are also two different genes, *mat1a* and *mat2a*, and that two genes show a tissue-specific pattern of expression (Gil et al. 1996).

Regulation of *sams* genes. In mammals, *mat1a* is expressed only in the adult liver whereas *mat2a* is expressed in a fetal liver and non-hepatic tissues. An interesting observation is that *mat2a* expressed in the fetal liver is replaced by *mat1a* upon maturation of this organ (Mato et al. 1997). Interestingly, the expression of two *sams* genes is switched in human liver cancer (Cai et al. 1996). Recent research has been focused on the mechanism for the switch of the *mat1a* to *mat2a* in the liver cancer, and silencing of *mat1a* by promoter methylation and histone acetylation in the hepatoma cells was suggested to be the mechanism (Torres et al. 2000). The other group investigated the 5′-flanking region of human *mat1a* gene with sequential deletion analysis of the promoter and they showed two DNA regions, –705 to –839 and –1111 to –1483, are involved in positive and negative gene regulation, respectively (Zeng et al. 2000). The *mat1a* also contains several consensus binding sites for CAAT enhancer binding protein (C/EBP) and hepatocyte-enriched nuclear factor (HNF), transcriptional factors important in liver-specific gene expression. In addition, consensus binding sites for activator protein-1 (AP-1), CAAT, glucocorticoid response element (GRE), E2F, c-Myc and v-Myb are also present. However, regulation of *sams* genes by methylation of the promoters has not been reported yet in other organisms.

In yeast, the expression of two *sams* genes is differently regulated depending on the growth stage (Thomas and Surdin-Kerjan 1991). The expression of *sam2* is dependent upon the growth stage whereas that of *sam1* remains constant during growth. The *sam2* gene is regulated by at least two mechanisms; one of which acts negatively on the expression of the other genes of SAM metabolism and another of which is responsible for this growth-dependent increase. Three *cis*-acting regulatory elements, which are involved in the transcriptional regulation of the sulfur metabolism-related genes (*met*) in *S. cerevisiae,* have been identified (Thomas and Surdin-Kerjan 1997). The ATGA(C/G)TCAT sequence is the DNA-binding site of the transcriptional activator of Gcn4p and related to general control of amino acid biosynthesis (Mountain et al. 1993). The CACGTG sequence is known as the binding site of a transcriptional complex (Cbf1-Met4p- Met28p) and required for derepression of *met* gene expression when intracellular concentration of SAM is low (Kuras et al. 1997). The last AAANTGTG element, which

is known as the binding site of Met31 and 32, is required for full repression of *met* genes at high intracellular concentration of SAM (Blaiseau et al. 1997). Interestingly, the third element (AAACTGTGG) exists in both *sam1* and *2* genes, whereas the second element (CACGTG) in only *sam2* gene (Thomas and Surdin-Kerjan 1997) although the relation between the *sams*-gene expression and these *cis*-elements have not been studied yet.

In *E. coli*, sulfur-metabolism-related genes are transcriptionally regulated by MetJ protein (repressor) and SAM (corepressor) (Shoeman et al. 1985). The basic interaction occurs between a homodimer of the 12-kDa MetJ repressor subunits and an 8-base pair sequence (AGACGTCT), which is known as a "Met box". The Met box is a tandem repeat that occurs between two and five times in natural operators and to which additional repressor dimers bind in a cooperative manner. Binding of the MetJ repressor to DNA is modulated by SAM, which markedly increases the affinity of MetJ for its target sequence (He et al. 1996).

CHAPTER 3
SPECIFIC AIMS

One of the unanswered questions on the amoeba/X-bacteria symbiosis is why the hosts become dependent on their symbionts. The expression of the *sams* gene is essential for the survival of cells. As a result of harboring X-bacteria, the expression of the host *sams* gene is completely suppressed in symbiont-bearing xD amoebae. Thus, xD amoebae apparently obtain the enzyme from another source. In supporting this view, xD amoebae still show about half the level of SAMS activity found in symbiont-free D amoebae in spite of the fact that they do not produce their own SAMS. Therefore, it is hypothesized that X-bacteria somehow suppress the expression of amoeba's *sams* and in turn provide the enzyme for their hosts. It appears that bacterial symbionts play a role in supplementing a function(s) formerly performed by the now-absent amoeba SAMS, forcing host amoebae to become dependent on symbionts themselves.

Specific Aim 1 in this study is to investigate the compensation of X-bacteria for the deficiency of SAMS in the host amoebae and the relationship between the dependence of hosts on their symbionts for survival and the compensation of SAMS by symbionts. Specific Aim 2 is to study the mechanism for the inactivation of amoeba *sams* gene by X-bacteria.

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PART II

CHARACTERIZATION OF *SAMS* GENES OF *AMOEBA PROTEUS* AND THE ENDOSYMBIOTIC X-BACTERIA

* Taeck J. Jeon and Kwang W. Jeon. 2003. *J. Eukaryot. Microbiol.*, 50(1):61-69.

CHAPTER 1

ABSTRACT

As a result of harboring obligatory bacterial endosymbionts, the xD strain of *Amoeba proteus* no longer produces its own S-adenosylmethionine synthetase (SAMS). When symbiont-free D amoebae are infected with symbionts (X-bacteria), the amount of amoeba SAMS decreases to a negligible level within four weeks, but about 47% of the SAMS activity, which apparently comes from another source, is still detected. Complete nucleotide sequences of *sams* genes of D and xD amoebae are presented and show that there are no differences between the two. Long-established xD amoebae contain an intact *sams* gene and thus the loss of xD amoeba's SAMS is not due to the loss of the gene itself. The open reading frame of the amoeba's *sams* gene has 1,281 nucleotides, encoding SAMS of 426 amino acids with a mass of 48 kDa and pI of 6.5. The amino acid sequence of amoeba SAMS is longer than the SAMS of other organisms by having an extra internal stretch of 28 amino acids. The 5'-flanking region of amoeba *sams* contains consensus-binding sites for several transcription factors that are related to the regulation of *sams* genes in *E. coli* and yeast. The complete nucleotide sequence of the symbiont's *sams* gene is also presented. The open reading frame of X-bacteria *sams* is 1,146 nucleotides long, encoding SAMS of 381 amino acids with a mass of 41 kDa and pI of 6.0. The X-bacteria SAMS has 45% sequence identity with that of *A. proteus*.

CHAPTER 2
INTRODUCTION

The xD strain of *Amoeba proteus* that arose from the D strain by spontaneous infection of X-bacteria in 1966 (Jeon & Lorch 1967) is now dependent on X-bacteria for survival, and each xD amoeba contains about 42,000 symbionts within symbiosomes. Newly infected xD amoebae become dependent on X-bacteria within 18 months (about 200 cell generations) (Jeon & Ahn 1978), and established xD amoebae die if their symbionts are removed. One of the unanswered questions on the amoeba-bacteria symbiosis is why the hosts become dependent on symbionts.

Recently, we found one clue that might shed light on the mechanism for the development of host dependence: symbiont-bearing xD amoebae do not transcribe the *sams* gene and no longer produce their own S-adenosylmethionine synthetase (SAMS) as a result of harboring X-bacteria (Choi et al. 1997). Meanwhile, xD amoebae still show about half the level of SAMS activity found in symbiont-free D amoebae, in spite of the fact that they do not produce their own SAMS. Thus, xD amoebae apparently obtain the enzyme from another source.

Earlier, it was found that D amoebae infected with X-bacteria lost amoeba SAMS within 50 days after infection (Choi et al. 1997). It was apparent that X-bacteria somehow suppressed the expression of amoeba's *sams* and in turn provided the enzyme for their hosts. Thus, it appears that bacterial symbionts play a role in supplementing a function(s) formerly performed by the now-absent amoeba SAMS, forcing host amoebae to become dependent on symbionts themselves. Since SAMS is an essential housekeeping enzyme, it follows that symbiont-bearing xD amoebae would lose viability if they lost X-bacteria.

As a part of our on-going work to elucidate the mechanism for the development of mutual dependence between symbionts and hosts, we sequenced and characterized *sams* genes of amoebae and of their endosymbionts. Here we report complete nucleotide sequences of the genes and some of their characteristics. Long-established xD amoebae contain the intact *sams* gene and there are no noticeable nucleotide differences between

sams genes of D and xD amoebae. Thus, the loss of xD amoeba's SAMS is not due to the loss of the gene itself but apparently by selective suppression of gene expression.

CHAPTER 3
MATERIALS AND METHODS

Amoebae. The D and xD strains of *A. proteus* were cultured in a modified Chalkley's solution (Jeon & Jeon 1975) in Pyrex baking dishes (35 × 22 × 4 cm). Amoebae were fed daily with axenically cultured and washed *Tetrahymena pyriformis* as food organisms (Goldstein & Ko 1976).

Amplification of X-bacteria *sams* gene. In order to clone the X-bacteria *sams* gene, we amplified X-bacteria DNA with degenerate PCR primers corresponding to DNA sequences encoding amino acids that were highly conserved among known SAMS (Yocum et al. 1996). The 5′-primer used corresponded to nucleotides for amino acids 139 -- 143 of the murine SAMS, Met-Phe-Gly-Tyr-Ala (CCCGGATCCATGTTYGGNTAYGC), and the 3′-primer was for residues 378 -- 382 (Tyr-Gly-His-Phe-Gly) (CCCGAATTCCCRAARTGNCCRTA) of the same SAMS. A *Bam*H1 restriction site was incorporated into the 5′-primer and an *Eco*RI site into the 3′-primer. The 750-bp amplified fragments were eluted and digested with *Bam*H1 and *Eco*RI. The digested DNAs were cloned into pBSKII$^+$ and sequenced (cf. Fig. 4A). We obtained a 719-bp stretch of X-bacteria *sams*, which was used for extending the 5′- and 3′-end sequences in subsequent experiments.

Construction of genomic libraries. The pBSKII$^+$ plasmid (5 μg) (Stratagene, La Jolla, CA) was digested with *Bam*H1 or *Eco*RI in 40 μl for 2 hr at 37 °C. To prevent recircularization of linearized plasmid DNAs, we dephosphorylated digested plasmid DNA (40 μl) with calf intestinal alkaline phosphates (CIAP) (Promega, Madison, WI) by adding 5 μl of CIAP 10 × reaction buffer plus 5 μl of diluted CIAP (0.01 U/μl) and by incubating for 30 min at 37 °C. Another aliquot of diluted CIAP was added and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 300 μl of CIAP stop buffer (10-mM Tris-HCl, pH7.5, 1-mM EDTA, pH 7.5, 200-mM NaCl and 0.5% SDS). The genomic DNA (1-2 μg) of *A. proteus* or symbiotic X-bacteria was digested with

*Bam*H1 or *Eco*RI in 10 μl for 1 hr at 37 °C. Dephosphorylated plasmid and genomic DNAs were extracted with an equal volume of phenol/chloroform/isoamyl alcohol and precipitated with 2.5 volumes of 100% ethanol. The pellet was washed with 70% ethanol and resuspended in sterile distilled water (dH_2O). The digested pBSKII$^+$ plasmid (0.1 -- 1 μg) and an equal amount of digested genomic DNA were ligated with 3 U of T4 DNA ligase (Promega) in 10 μl for 1.5 hr at room temperature.

Amplification of 5′- and 3′-end genomic DNA by PCR. Ligated DNA in 5 μl was amplified with two primers, one gene specific and the other plasmid specific. PCR was performed using 5 or 10 μl of the ligated DNA as a template, 0.5 μM of the gene-specific primer (forward or reverse), 0.5 μM of the plasmid-specific primer (T7 primer; 5′-TAATACGACTCACTATAGGGCGA-3′), 200-μM dNTPs, and 5 U of *Taq* DNA polymerase (Promega) in 50 μl. PCR was carried out in 35 cycles for 1 min at 94 °C, 1 min at 55 °C, and for 1.5 min at 72 °C, respectively.

In PCR for extending 5′-end sequences, a reverse sequence located inside the known sequence was used as a gene-specific primer. In the case of 3′-end extension, a forward sequence located inside the known sequence was used. The T7 primer was used as the plasmid-specific primer in both experiments.

For extending a partial D-amoeba *sams* gene (Choi et al. 1997), we digested amoeba genomic DNA and pBSKII$^+$ with *Eco*RI, ligated them and amplified the ligated mixture with a gene-specific reverse primer *SAM1* (5′-GTCCTCTGCTGACTGCTTCG-3′), located at nt 52 -- 33 in D-amoeba *sams*, and a plasmid-specific T7 primer. As a result, we obtained 580-bp fragments, subcloned them into the pGEM-T Easy vector, and sequenced them. The extended fragments contained a 546-bp upstream stretch not present in the amoeba *sams* used as the starting sequence.

For extending the 5′-end of X-bacteria *sams*, we digested X-bacteria DNA and pBSKII$^+$ with *Bam*H1, ligated them, and amplified the ligated product with *Xbac2* (5′-CTGATAGAATGTGGCTCTGC-3′), located at nt 569 – 550 in the 719-bp sequence of X-bacteria *sams* we had cloned (cf. Fig. 4), and T7 primers. Resulting 1.5-kb fragments (cf. Fig. 4B) were cloned into pGEM-T Easy vector and sequenced. The fragments

contained a 1,283-bp sequence, of which 714 bp represented a new upstream extension that contained the transcription-start point (TSP) and promoter site. For extending the 3′-end of X-bacteria *sams*, we digested X-bacteria DNA and pBSKII$^+$ with *Eco*RI, and then ligated them. The ligated X-bacteria DNA/pBSKII$^+$ fragments were amplified with *Xbac1* (5′-GATCAGCCACAAAGATCTTC-3′), located at nt 213 – 232, and T7 primers (cf. Fig. 4C). We cloned the resulting 800-bp fragments into pGEM-T Easy vector and sequenced. The cloned fragments contained a 267-bp downstream sequence that included the stop codon. Thus, by extending the 5′- and 3′-end sequences of the X-bacteria *sams* gene, we obtained 1.7-kb fragments.

Analysis of PCR products. The PCR-amplified DNA was analyzed by electrophoresis in 0.8% agarose gel and fragments were eluted by using the GENE Clean Kit II (Bio101, Vista, CA). For selecting gene-specific DNA fragments, eluted DNA in 1 μl was further amplified by PCR under the same conditions as described above. Two rounds of PCR were performed with the two primers for comparison. Fragments amplified by PCR with both primers were subcloned into the pGEM-T Easy Vector (Promega) and sequenced. When many fragments were found in a gel, eluted fragments were identified by Southern blotting using gene-specific probes. Homology searches were performed by the BLAST program on the NCBI file server (Altchul et al. 1990). Multiple sequence alignments were performed with the PILEUP and PRETTYBOX programs of the GCG sequence analysis package. Further protein and DNA analyses were performed using the GCG program and the TRANSFACFind search engine (Heinemeyer et al. 1999).

Comparing *sams* genes of D and xD amoebae. In order to compare *sams* genes of D and xD amoebae, we determined the nucleotide sequence of xD amoeba's *sams* as follows. We obtained a portion (nt –530 – 1212) of xD amoeba's *sams* by amplifying the genomic DNA of xD amoebae by PCR and sequenced the product. A forward primer located at nt –530 -- –510 (5′-CGAATGTAGAAATCATTGGAG-3′) and a reverse primer located at nt 157 – 138 (5′-ATCGTCGTCTCGAGAGCCAC-3′) were used for amplifying the 5′-end sequence of xD-amoeba *sams*. For amplifying the coding sequence

of xD-amoeba *sams*, we used two primers, located at nt 106 – 126 (5′-CAAGCCGAGAAGAAATGCAAG-3′) and at nt 1212 – 1189 (5′-CTCAACATATTTGATACGATCGGG-3′), respectively. Then we compared *sams* genes of D and xD amoebae using GCG programs in order to check if the absence of SAMS in xD amoebae was attributable to the loss of the gene itself.

Primer extension analysis. Total RNA was extracted from *A. proteus* and *E. coli* transformed with X-bacteria *sams* using the SV Total RNA Isolation System (Promega). An antisense oligonucleotide primer complementary to nt 33 -- 55 relative to the translation initiation site of the amoeba *sams* was end-labeled with [γ-^{32}P]ATP using a T4 polynucleotide kinase. In the case of X-bacteria *sams*, an antisense oligonucleotide primer complementary to nt –109 -- –90 from the translation initiation codon of X-bacteria *sams* was used. The transcription-start site was determined by primer extension (Ausubel et al. 1992). An aliquot of labeled primer (20 ng) was mixed with 50 μg of RNA. The mixture was denatured by heating for 3 min at 100 °C, followed by incubation for 1.5 h at 65 °C, and then allowed to cool down slowly to 37 °C. Reverse transcription was carried out with 40 -- 50 U of AMV reverse transcriptase or 200 -- 400 U of MMLV reverse transcriptase (Promega). The primer-extended products were analyzed by electrophoresis in a 6% acrylamide/urea sequencing gel.

RNase protection assay. An antisense primer complementary to nt 84 -- 63 of amoeba *sams* was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The end-labeled antisense primers were used in amplifying 5′-end fragments together with another sense primer located at nt –255 to –236. Amplified 340-bp fragments were purified and used in the RNase protection assay. Total RNA (100 μg) from D amoebae was hybridized with end-labeled probes in the hybridization solution (80% formamide, 0.4-M NaCl, 20-mM HEPES, pH 6.5) for 10 min at 75 °C and then for 14 h at 37 °C. After incubation, 240 μl of diethyl-pyrocarbonate-treated dH_2O and 30 μl of S1 nuclease buffer (0.3-M sodium acetate, pH 4.6, 5-M NaCl, 10-mM $ZnSO_4$, and 50% glycerol) were added to the hybridization mixture. The RNA was digested with S1 nuclease (100 U) for 30 min at 37

°C. Labeled probes were extracted with phenol and precipitated with ethanol. Remaining DNA-RNA hybrid fragments were analyzed as described above for primer extension analysis. To determine the TSP of X-bacteria *sams*, we used an end-labeled antisense primer located at nt 20 -- 39 of X-bacteria *sams* and another sense primer for amplifying 5′-end fragments. Amplified 387-bp fragments were hybridized with total RNA (100 μg) from *E. coli* transformed with X-bacteria *sams* and then analyzed as described above.

Recombinant proteins and Western blot analysis. Amoeba SAMS was produced as a fusion protein in *E. coli* BL21 (DE3) transformed with *sams*/pET30a, constructed from *sams*/pBSK$^-$ that had been obtained from a cDNA library of D amoebae. For the purpose, 838-bp fragments (nt 145 -- 982 from the translation-initiation codon) of the coding sequence of amoeba *sams* in *sams*/pBSK$^-$ were subcloned into a *Kpn*I/*Sac*I site of the pET30a vector to be expressed in *E. coli* BL21 (DE3). The fusion protein contained a His-tag stretch sequence. Recombinant amoeba SAMS was located in inclusion bodies and purified by metal affinity chromatography on His-bind Resin (Novagen) in the presence of 6-M urea.

For Western blot analysis, an anti-SAMS mouse antibody raised against recombinant amoeba SAMS was used. For its production, 200 μg of purified recombinant SAMS were mixed with an equal volume of Freund's complete adjuvant and injected intraperitoneally into 6-week-old female BALB/c mice. The first injection was followed by three booster injections at two-week intervals, and the final injection was given three days before mice were killed and sera were taken.

To determine the amount of SAMS in D amoebae infected with X-bacteria, we isolated X-bacteria from xD amoebae and introduced them into D amoebae (Kim et al. 1994). We collected the whole-cell proteins of infected amoebae every 4 days for 4 weeks after infection and determined the amount of SAMS by measuring the density of transferred protein bands on membranes immunoblotted with an anti-SAMS mouse polyclonal antibody (Choi et al. 1997). To measure the density of immunoblotted SAMS bands, we used the HP ScanJet Iicx Scanner (Hewlett-Packard) and ImageTool software

(University of Texas Health Science Center, San Antonio). The results were normalized by setting the amount of SAMS found in control D amoebae as 1.

Northern blotting and RT-PCR. To determine the amounts of transcripts of amoeba *sams* after infection of D amoebae with X-bacteria, we prepared total RNA using the SV Total RNA Isolation System (Promega) from samples collected every 4 days for 4 weeks after infection as described above. We carried out Northern-blot analysis by the usual method (Ausubel et al. 1992). For RT-PCR, total RNA (1 μg) from xD amoebae was reverse-transcribed with AMV reverse transcriptase (Promega) and random hexamers. PCR was performed with X-bacteria *sams* primers and reaction products were analyzed in 1% agarose gel. In performing RT-PCR, we used a forward primer, located at nt –46 -- –25, and a reverse primer, which was located at nt 935 -- 916. Amoeba's *myosin* primers were used as internal controls.

CHAPTER 4
RESULTS

First, in order to follow the disappearance of amoeba SAMS from newly infected xD amoebae in more detail, we infected D amoebae with X-bacteria and determined the amount of SAMS at intervals. The amount of amoeba SAMS decreased to a negligible level within four weeks following infection (Fig. 1A), a few weeks sooner than reported previously (Choi et al. 1997). In addition, the amount of *sams* mRNA detected by Northern blotting (Fig. 1B) decreased in a similar manner as did SAMS. In a preliminary study by run-on assays using D- and xD-amoeba nuclei, we found the inactivation of amoeba *sams* by X-bacteria to occur at the transcriptional level (T. Jeon and K. Jeon, unpublished).

Nucleotide sequences of *sams* genes of D and xD amoebae. We obtained the complete nucleotide sequence of the D-amoeba *sams* gene by extending 5′-end of a partial sequence of the gene, that had been obtained earlier by screening an amoeba cDNA library and which lacked the 5′-end sequence (Choi et al. 1997). The 5′-end sequence of D-amoeba *sams* and the deduced amino acid sequence are shown in Fig. 2A. The newly extended 546-bp 5′-end portion contained a TSP, as determined by primer extension using an antisense oligonucleotide primer complementary to nt 33 -- 55 relative to the translation-initiation site of the amoeba *sams* gene (Fig. 2B). We found two major transcription-start sites at nt –28 (T) and –30 (C) from the translation-initiation codon by primer extension using the AMV reverse transcriptase. However, in primer-extension experiments using MMLV reverse transcriptase, in which the RNase H activity was weaker than that of the commonly used AMV reverse transcriptase, other extended fragments ending with T at nt –63 and –56 were found. To confirm these data, we performed S1-nuclease mapping (Fig. 2C) using end-labeled 340-bp fragments and found 140-bp fragments to be protected from S1-nuclease digestion. The site of the protected fragments matched with the position at nt –56 from the translation-initiation site. Sequence analysis revealed a TATA box located at the nt position of –112 -- –103 and a

cap signal (CA followed by a number of pyrimidines) (Bucher 1990) at nt –72 -- –65 (Fig. 2A). The ORF of amoeba's *sams* gene had 1,281 nucleotides, encoding SAMS of 426 amino acids with a mass of 48 kDa and pI of 6.5, as determined by ExPASy molecular biology server.

Transcription-factor-binding sites were searched by using the Transcription Factor Search method (URL: http://www.pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). The 5′-flanking region of amoeba's *sams* contained several consensus-binding sites known for CAAT enhancer-binding protein (C/EBP), activator protein-1 (AP-1), cAMP response element-binding protein (CRE-B), c-Myb, and c-Myc. In addition, we found two consensus-binding sites for the Met box, and one site each for the AAANTGTG and CACGTG element (Fig. 2A).

When the N-terminal amino acids of D amoeba's SAMS were compared with those of other organisms, the first methionine of amoeba SAMS was located at the same position as that of SAMS of several other organisms, including *Acanthamoeba castellanii* and *Oryza sativa,* and one amino acid before that of *Leishmania infantum, Pisum sativum, Hordeum vulgare,* and *Caenorhabditis elegans* (Fig. 2D). The N-terminal amino-acid sequence of amoeba SAMS shared many identical residues with SAMS of other organisms. Compared with SAMS of most other organisms that have 380 -- 400 amino acids, amoeba's SAMS was longer because it had an extra internal stretch of 28 amino acids.

Next, we determined the nucleotide sequence of the xD amoeba's *sams* gene and compared it with that of D-amoeba *sams* using GCG programs in order to check if the absence of SAMS in xD amoebae was contributable to the loss of the gene itself. There was no noticeable nucleotide difference between the two genes.

Nucleotide sequence of X-bacteria *sams*. We obtained a 1.7-kb fragment of X-bacteria *sams* (Fig. 3) by extending both ends of a 719-bp stretch of X-bacteria *sams* (Fig. 4), that had come from an amplified X-bacteria DNA with degenerate PCR primers corresponding to DNA sequences encoding amino acids that were highly conserved

among known SAMS (Yocum et al. 1996) (Fig. 4A). Other PCR-amplified fragments of the *sams* gene of X-bacteria detected in agarose gels are shown in Fig. 4B,C.

In order to ensure that the DNA sequence was that of X-bacteria, we analyzed genomic DNAs of D and xD amoebae and of X-bacteria by Southern blotting after digesting genomic DNAs with *Eco*RI. We found a common band in the DNA of xD amoebae and X-bacteria (data not shown). Also, we carried out Northern blotting and RT-PCR using total RNAs isolated from D and xD amoebae to check the expression of X-bacteria *sams*. As a result of RT-PCR, we detected an expected band in the cDNA of xD amoebae (Fig. 5). For excluding any potential contamination of genomic DNA, RNAs used for reverse transcription were also amplified without reverse transcription, but no band was amplified (data not shown). In Northern blots using total RNAs of both D and xD amoebae, no positive band was found. The results indicated that X-bacteria *sams* was transcribed at a very low level.

The TSP of X-bacteria *sams* was determined by primer extension and S1-nuclease mapping (Fig. 6). Two extended fragments with C at nt –127 and –128 from the translation initiation codon were observed (Fig. 6A). The results were confirmed by S1-nuclease protection assay (Fig. 6B), which showed the fragments ending with nt –127 (arrow) to be protected from S1 nuclease digestion. Our S1-nuclease mapping showed that 166-bp fragments were protected from S1-nuclease digestion and that the site of protected fragments matched with the position at nt –127 from the translation-initiation site. A putative promoter site (–10 and –35 elements) was found above TSP (Fig. 3, shaded) (Nair & Kulkarni 1994). The X-bacteria *sams* gene had an ORF of 1,146 nt (cf. Fig. 3), encoding 381 amino acids, and it was similar to that of other organisms. The X-bacteria SAMS had a mass of 41 kDa, with pI 6.0, as determined by ExPASy molecular biology server. The X-bacteria SAMS contained a consensus ATP-binding motif (GAGDQG) at amino acid residues 116 --121 (Fig. 7), a motif conserved in all identified SAMS; (Choi et al. 1997). The glycine-rich nanopeptide (GGGAFSGKD) was also found in X-bacteria SAMS. In X-bacteria and *S. aureus*, the Ala residue was replaced with Cys.

When the deduced amino-acid sequence of X-bacteria SAMS (XB) was aligned with that of other SAMS homologs, varying degrees of sequence identity were found

(Fig. 7). The sequence identity of X-bacteria SAMS with that of *A. proteus* was 45%, while it was 65% with *E. coli*, 69% with *Haemophilus influenza*, and 61% with *S. aureus*.

CHAPTER 5
DISCUSSION

Our results show that the amoeba's *sams* gene is inactivated by symbiotic X-bacteria and that no transcript of *sams* or its product is found in xD amoebae. The removal of symbiotic X-bacteria does not restore the expression of amoeba *sams* as checked by Western blotting (T. Jeon and K. Jeon, unpublished), and hence the inactivation of amoeba *sams* appears to be irreversible. Meanwhile, xD amoebae possess some activity of SAMS compared with D amoebae and thus the lack of amoeba's SAMS or SAM in the host amoebae is apparently compensated by symbionts. That may be the reason for host amoebae to become dependent on their symbionts for survival.

The SAMS enzyme catalyzes the formation of S-adenosylmethionine (SAM) from methionine and ATP, and the catalysis is the only known route of biosynthesis of the primary biological alkylating agent (McQueney et al. 2000). Being the most important biological methyl donor, SAM is known to be required for the survival and continued growth of various organisms, including *Pneumocystis carinii* (Merali et al. 2000) and yeast (Hilti et al. 2000). Other roles of SAM appear to vary in different organisms. For example, in *E. coli*, a lowered level of SAM causes C-to-T mutations (Macintyre et al. 2001). In LPS-stimulated murine macrophages, SAM lowers the amount of tumor necrosis factor alpha (TNF-α) in a dose-dependent manner (Watson et al. 1999), and SAM has protective actions against damage in hepatic cells (Carretero et al. 2001; Holecek et al. 2000; Jeon and Lee 2001; Lee & Lee 2000).

In this study, we sequenced *sams* genes of amoebae and of X-bacteria as a part of our investigation on the apparent compensation by X-bacteria for the deficiency of SAMS in symbiont-bearing amoebae and resulting dependence of amoebae on their symbionts for survival. It is not yet clear how X-bacteria inactivate amoeba's *sams*, but some possible mechanisms could be considered. The loss of xD amoeba's SAMS is not attributable to the loss or modification of the xD amoeba's *sams* gene itself since xD amoebae have the *sams* gene and it is not different from D amoeba *sams*. It is possible that the two kinds of plasmids present in X-bacteria, pHJ11 and pHJ12, might be

involved by being transferred into amoeba nuclei when amoebae are infected with X-bacteria (Choi et al. 1997). The bacterial DNA could be integrated into an amoeba's gene indirectly involved in the regulation of amoeba *sams* and thus may cause the inactivation of the *sams* gene.

It is also possible that amoeba's *sams* is inactivated by methylation as in the case of hepatoma cells in which *MAT1A* was recently found to be silenced by promoter methylation and histone acetylation (Thomas & Surdin-Kerjan 1997; Torres et al. 2000). Results of our preliminary studies have shown that some of the 11 Dam methylation sites present in xD amoeba *sams* are methylated as checked by using methylation-sensitive restriction enzymes (T. Jeon and K. Jeon, unpublished). There are many CpG sequences present in the 5′-end region of amoeba *sams*, but their methylation pattern was not different between *sams* genes of D and xD amoebae as checked by bisulfite-genomic sequencing. Amoeba's SAMS is longer than other SAMS by having an extra internal stretch of 28 amino acids, but its significance is not known.

Involvement of specific transcription factors and/or binding sites might also be considered. The 5′-flanking region of amoeba *sams* contains two Met boxes and one site each of AAANTGTG and CACGTG elements (cf. Fig. 2). In yeast, the CACGTG and AAANTGTG elements are related to the transcriptional regulation of the sulfur amino acid (Mountain et al. 1993; Blaiseau et al. 1997) and are shown to be present in *sams* genes *of S. cerevisiae* (Thomas & Surdin-Kerjan 1997). The CACGTG sequence is known to be the binding site for a transcriptional complex (Cbf1- Met4p- Met28p) and is required for the de-repression of *met* gene expression when the intracellular concentration of SAM is low. The AAANTGTG element is thought to be the binding site for Met31 and Met32, and it is required for a full repression of *met* genes at high intracellular SAM concentrations. In *E. coli*, several genes involved in methionine biosynthesis are transcriptionally regulated by MetJ protein (repressor protein) and SAM (corepressor). The basic interaction occurs between a homodimer of the 12-kDa MetJ repressor subunits and an 8-bp sequence (AGACGTCT), known as the "Met box" (He et al. 1996; Shoeman et al. 1985). The two consensus-binding sites present in the 5′-flanking region of amoeba *sams* may play a role in controlling the gene expression.

It was not clear why several TSPs (at nt –28, –30, –56, and –63) were found in the amoeba's *sams* gene in primer extension experiments (cf. Fig. 2), whereas S1-nuclease mapping showed only one TSP located nt –56. It is suspected that the secondary structure of mRNAs of amoeba *sams* produced several extended fragments during primer extension. Sequence analysis showed a TATA box in amoeba *sams*, located at nt –112 -- –103, and a cap signal (CA followed by a number of pyrimidines) at –72 -- –65 (cf. Fig. 2). The TSP detected by primer-extension and S1-nuclease mapping is located approximately 50 nt downstream of the predicted consensus TATA box and 10 nt downstream of the cap signal. These data contrast with the known facts that the TATA box is usually found in most protein-encoding eukaryotic genes centered at nt 25 -- 30 upstream from TSP and that the preferred region of the cap signal is centered at nt 2 downstream from the transcription-initiation site (Bucher 1990). However, the location of the transcription-start site of amoeba's *sams* is similar to that of the yeast *sam1* gene. In most yeast genes, the TATA box has been found between nt 35 and 180 upstream from the mRNA start site. The transcription-initiation site and TATA box of yeast *sam1* are located at nucleotide positions –62 and –119, respectively (Thomas & Surdin-Kerjan 1997).

The nucleotide sequences near the TSP of X-bacteria *sams* were similar to those of X-bacteria's *s29x* gene (Pak & Jeon 1996), in which transcription was found to be initiated at nt –142 upstream from the translation-initiation codon. In our experiments, we had to use RNA isolated from *E. coli* transformed with X-bacteria *sams* because X-bacteria could not be cultured outside amoebae. Thus, it was not clear if the identified TSP actually functioned in vivo.

In a preliminary study, we carried out in-vitro transcription of amoeba's *sams* using nuclear extracts of amoebae and found an expected band (T. Jeon and K. Jeon, unpublished). Thus, the amoeba's *sams* gene appears to contain all components for the expression of the gene. Results of the RT-PCR analysis using xD-amoeba's total RNA suggested that X-bacteria *sams* was transcribed at a very low level inside xD amoebae (cf. Fig. 5). However, further study is needed to confirm the results using RNA isolated from X-bacteria. Studies are in progress to elucidate the mechanism for the compensation

by X-bacteria for the deficiency of SAMS in host amoebae and subsequent development of host's dependence on symbionts. These studies include detection of methylation of xD amoeba *sams*, DNase I footprinting analysis and in-vitro transcription using amoeba *sams* to determine which transcription factors or binding sites are involved in the regulation of amoeba's *sams* genes.

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APPENDIX

Fig. 1. A Coomassie blue-stained gel, blots and graphs to show the decrease in the amount of amoeba SAMS and of *sams* mRNA in newly infected D amoebae. **A.** Immunoblots (upper panel) of D amoeba SAMS following infection with X-bacteria. As a control for loading, the Coomassie-blue-stained SDS-PAGE gel (middle panel) was used. Each point in the graph (lower panel) represents the amount of amoeba SAMS, as measured by the density of transferred protein bands in the immunoblot. Each point represents an average of measurements from three separate experiments. Bars indicate standard deviations. **B.** Northern blots of D amoeba *sams* following infection with X-bacteria and of *myosin* mRNA (used as an internal control). Each bar in the graph represents the density of the amoeba *sams* mRNA normalized for the density of *myosin* mRNA. For probing amoeba-*sams* mRNA in Northern blotting, 1.1-kb fragments (nt 106 -- 1212) of amoeba's *sams* amplified by PCR were labeled with [^{32}P]-dCTP by the Prime-a-Gene labeling system (Promega). For probing *myosin* mRNA, 0.5-kb fragments (nt 989 -- 1455) of amoeba *myosin* (Oh & Jeon 1998) were amplified and labeled with [^{32}P]-dCTP.

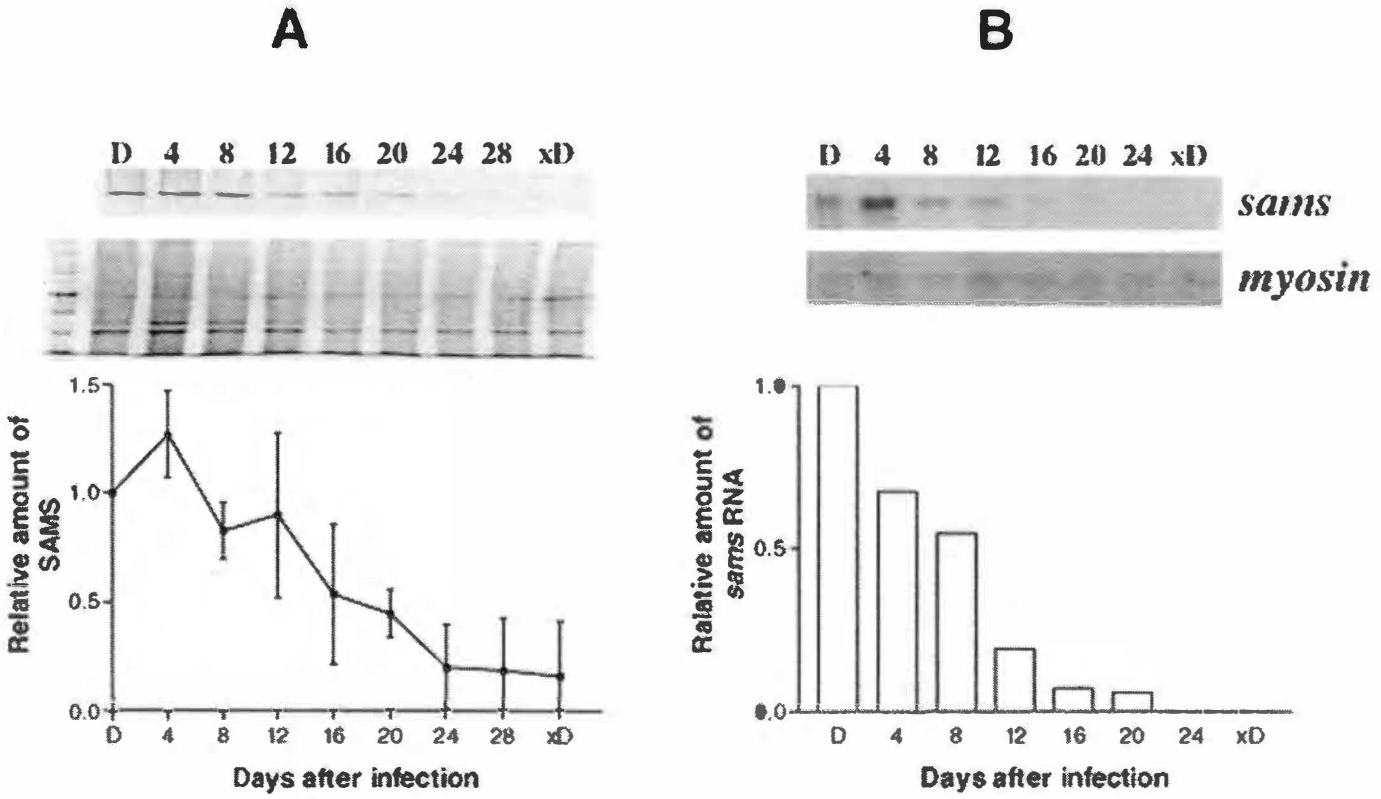
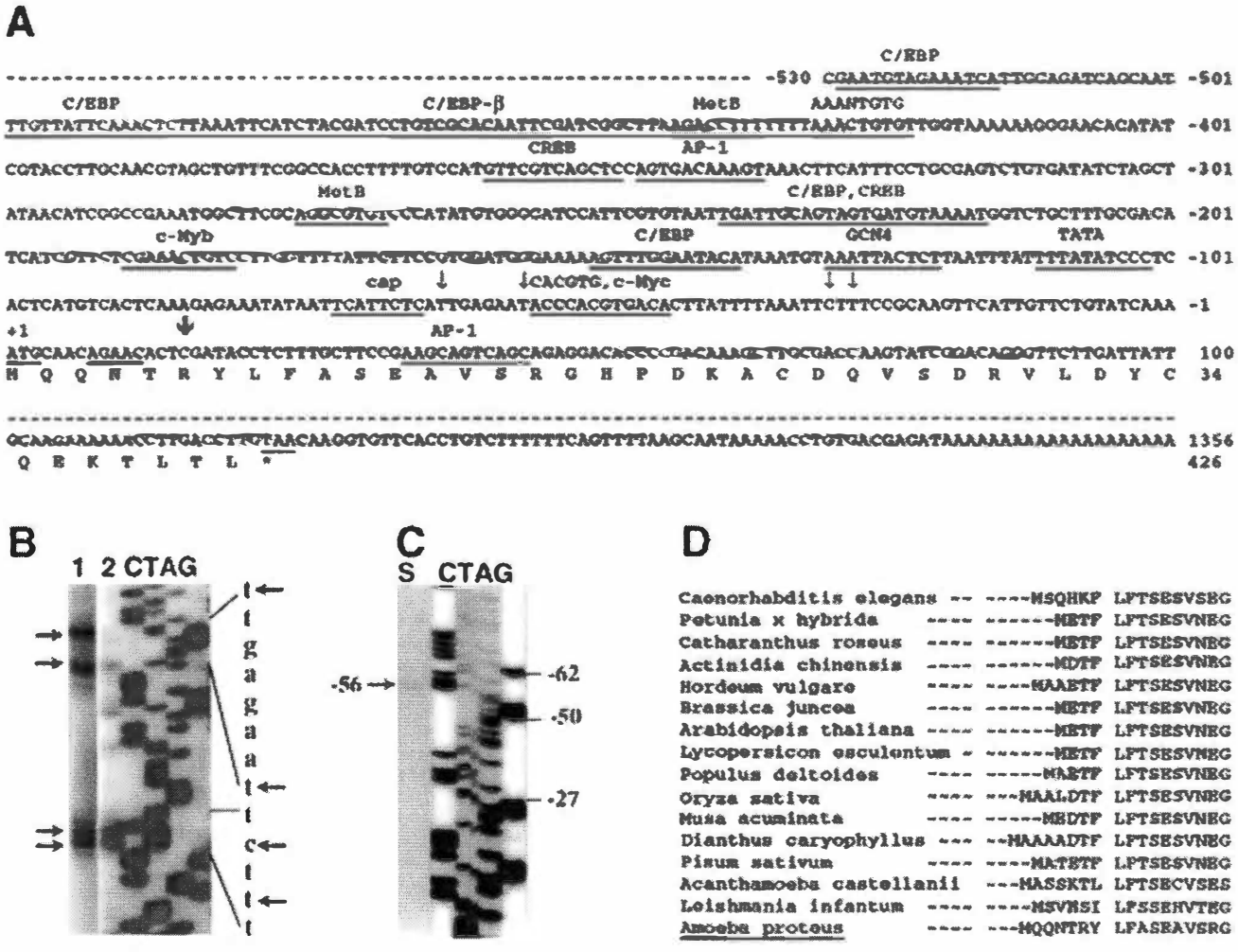

A
D 4 8 12 16 20 24 28 xD
Relative amount of SAMS
1.5
1.0
0.5
0.0
D 4 8 12 16 20 24 28 xD
Days after infection
B
D 4 8 12 16 20 24 xD
sams
myosin
Relative amount of sams RNA
1.0
0.5
0.0
D 4 8 12 16 20 24 xD
Days after infection

Fig. 2. The nucleotide sequence of amoeba's *sams* and N-terminal amino acid sequences of SAMS homologs. **A.** The 5'-end sequence of D-amoeba *sams* and the deduced amino acid sequence. The putative regulatory elements are indicated by letters above the underlined nucleotides. The thick arrow (⬇) indicates the first nucleotide of the newly extended DNA stretch. The transcription-start points are marked with thin arrows (↓↓). **B.** Results of primer extension for amoeba *sams*. Lane 1, Results of primer extension using MMLV reverse transcriptase. Lane 2, Results of primer extension using AMV reverse transcriptase. Arrows point to the transcription-start sites (T at nt –63, –56, and –28 and T at nt –28, and C at nt –30). The sequencing ladder (Lanes CTAG) was prepared by sequencing amoeba's *sams* using the same primers used for primer extension. **C.** Results of S1-nuclease mapping. Lane S, Results of S1-nuclease protection assay. The arrow indicate the position of sequences protected from S1 nuclease digestion, ending with nt –56. The sequencing ladder for CTAG lanes was prepared by sequencing amoeba *sams* using the same primer used in primer extension. **D.** Alignment of N-terminal amino-acid sequences of SAMS homologs. The first methionine in amoeba SAMS is located at the same position as in that of *Acanthamoeba castellanii* and *Orvza sativa*, and one amino acid before that of *Leishmania infantum*, *Pisum sativum*, *Hordeum vulgare*, and *Caenorhabditis elegans*.


A
C/EBP
-530
-501
C/EBP
C/EBP-β
MotB
AAANTGTG
-401
CREB
AP-1
-301
MotB
C/EBP,CREB
-201
c-Myb
C/EBP
GCN4
TATA
-101
cap
CACGTG, c-Myc
-1
+1
AP-1
100
34
1356
426
B
1 2 CTAG
C
S CTAG
-56
-62
-50
-27
D
Caenorhabditis elegans
Petunia x hybrida
Catharanthus roseus
Actinidia chinensis
Hordeum vulgare
Brassica juncea
Arabidopsis thaliana
Lycopersicon esculentum
Populus deltoides
Oryza sativa
Musa acuminata
Dianthus caryophyllus
Pisum sativum
Acanthamoeba castellanii
Leishmania infantum
Amoeba proteus

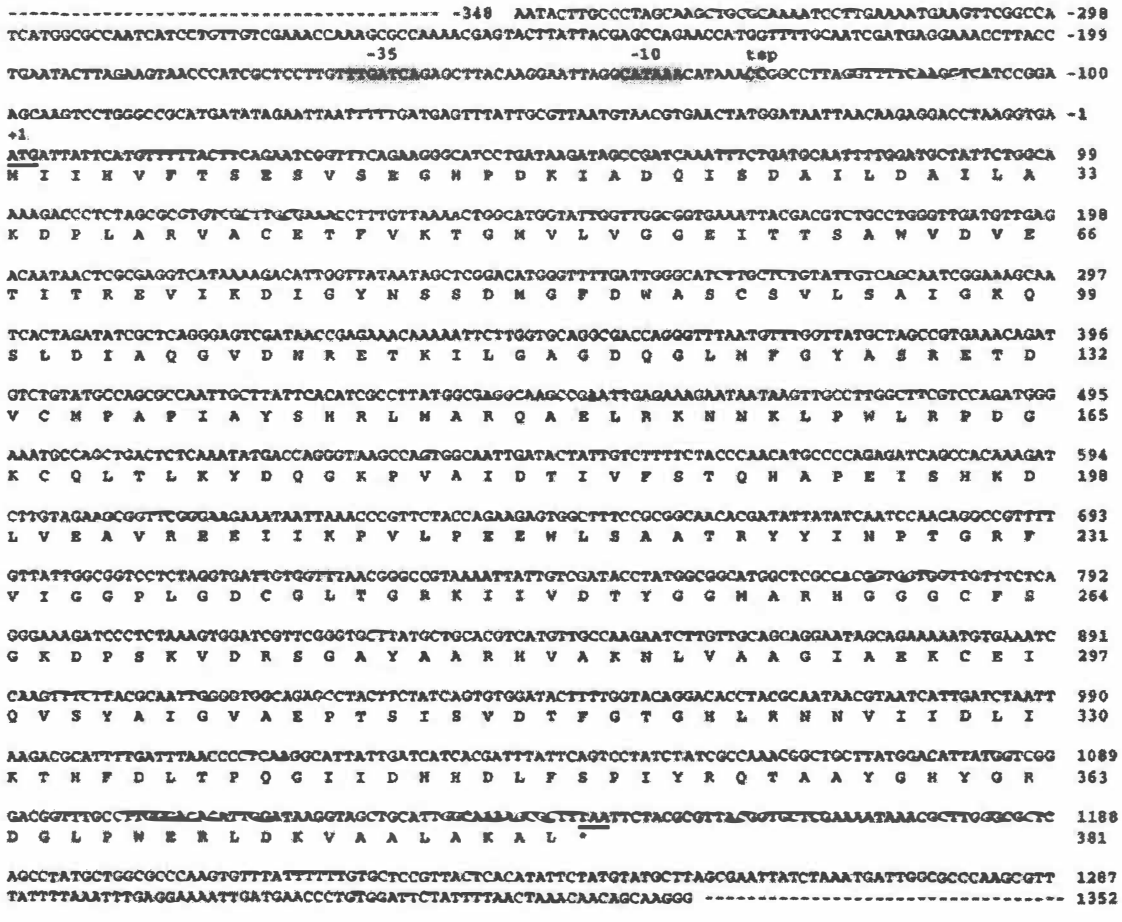


Fig. 3. Complete nucleotide and deduced amino-acid sequences of the X-bacteria *sams* gene. The ORF of X-bacteria *sams* is 1,146 nt long and encodes SAMS of 381 amino acids. The putative promoter region (−35 and −10) and tsp are highlighted, and the start and stop codons are underlined.

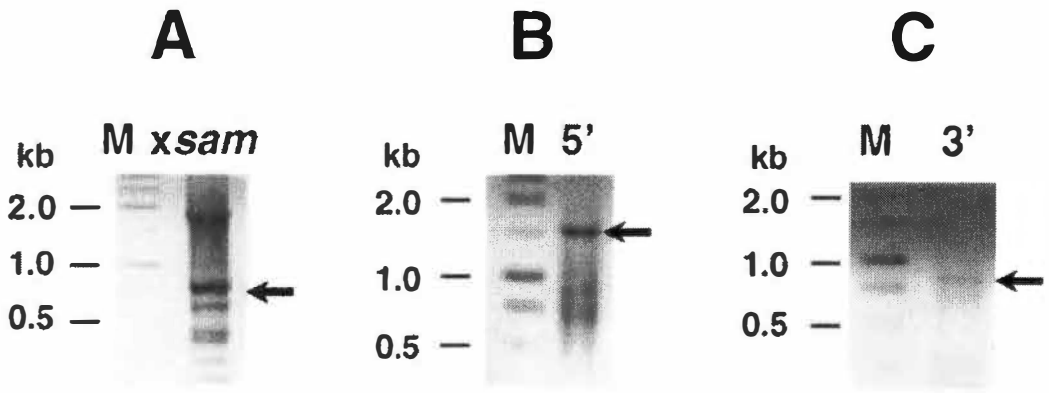


Fig. 4. Agarose gels showing PCR-amplified 5'- and 3'-end fragments of the *sams* gene of X- bacteria. **A.** Cloned X-bacteria *sams*, obtained by amplifying X-bacteria DNA with PCR primers corresponding to amino-acid sequences highly conserved in known SAM synthetases (Yocum et al. 1996). Fragments of 750 bp (arrow) were cloned into the PBSKII$^+$ vector and sequenced later. Lane M, 1-kb ladder used as the size marker in all figures. **B.** Extended 1.5-kb 5'-end fragments of X-bacteria *sams* (arrow), obtained by amplifying ligated X-bacteria DNA and PBSKII$^+$ after digestion with *Bam*H1. Gene-specific reverse *Xbac2* and plasmid-specific (T7) primers were used for the amplification. **C.** Extended 800-bp 3'-end fragments of X-bacteria *sams* (arrow) obtained by amplifying ligated X-bacteria DNA and PBSKII$^+$ after digestion with *Eco*RI. Gene-specific forward *Xbac1* and plasmid-specific (T7) primers were used for the amplification.

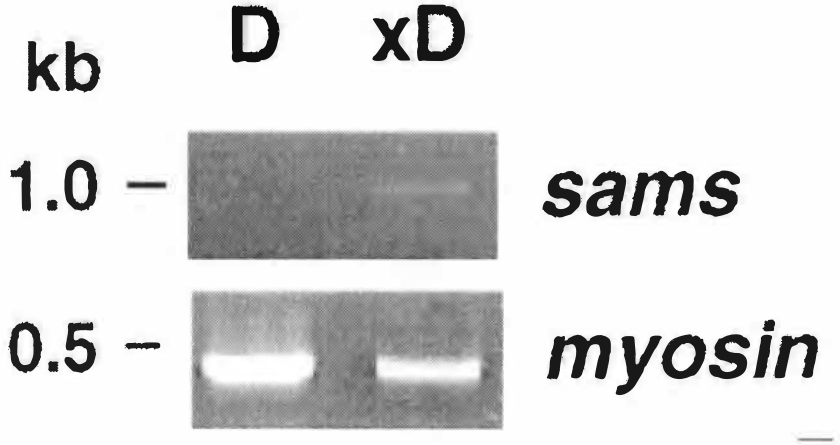


Fig. 5. Results of RT-PCR of D and xD amoeba RNAs with primers specific to X-bacteria *sams*. The reaction products were analyzed in 1% agarose gel. Total RNAs from D and xD amoebae were reverse-transcribed with random hexamer and subsequent PCR was performed with the X-bacteria *sams* primer or a *myosin* primer. The amoeba *myosin* gene was used as a control.

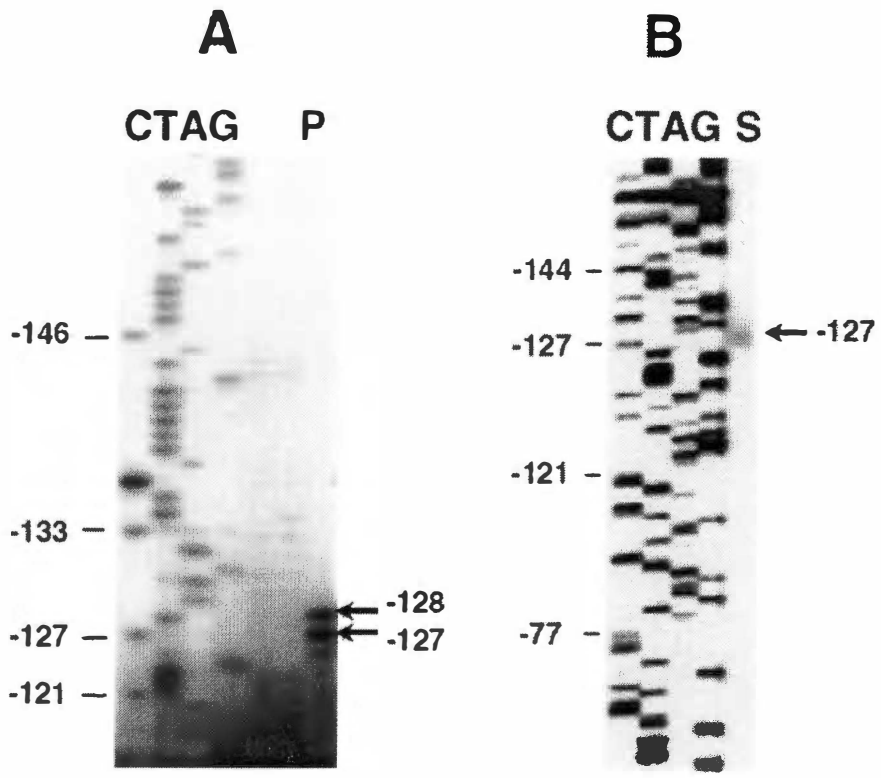


Fig. 6. Results of primer extension and S1 nuclease digestion of X-bacteria *sams*. **A.** Lane P, Results of primer extension experiments. Arrows indicate the positions of extended products, ending with nt −128 and −127, respectively. The sequencing ladder for CTAG lanes was prepared by sequencing X-bacteria *sams* with a primer complementary to nt 45 -- 64. A [^{32}P]-end-labeled primer complementary to nt −110 -- −91 of X-bacteria *sams* was used in extending the primer. **B.** Results of S1-nuclease mapping shown in Lane S. The arrow indicates the fragments ending at nt −127 and protected from S1 nuclease digestion. The sequencing ladder for CTAG lanes was prepared by sequencing X-bacteria *sams* with a primer complementary to nt −86 -- −67. A [^{32}P]-end-labeled primer complementary to nt 20 -- 39 of X-bacteria *sams* was used in the amplification of the 5'-end fragments, together with another sense primer from nt 348 -- 329. Amplified 387-bp fragments were used in the RNase protection assay.

Fig. 7. Alignment of deduced amino-acid sequences of X-bacteria SAMS (XB) with SAMS of other organisms. Periods represent amino acids identical to those of MetX of *E. coli*, and dashes show gaps inserted for an optimal alignment of amino acids. The conserved motif, GAGDQG, and glycine-rich nanopeptide are shaded. The sequence identity of X-bacteria SAMS with that of *A. proteus* was 45%, while it was 65% in *E. coli*, 69% in *Haemophilus influenza*, and 61% in *S. aureus*, respectively. EC, *E. coli*; HI, *Hmaemophilus influenzae*; SA, *Staphylococcus aureus*; AP, *Amoeba proteus*.

PART III

GENE SWITCHING IN *AMOEBA PROTEUS* CAUSED BY ENDOSYMBIOTIC BACTERIA

* This part is to be submitted for publication as Taeck J. Jeon and Kwang W. Jeon. 2003.

CHAPTER 1

ABSTRACT

The S-adenosylmethionine synthetase (SAMS) is the enzyme that catalyzes the synthesis of S-adenosylmethionine (SAM), a major methyl donor in cells. The expression of amoeba-*sams* (*sams1*) gene is completely suppressed within four weeks after infection with their symbionts (X-bacteria). However, symbiont-bearing xD amoebae still show approximately half the level of SAMS activity found in symbiont-free D amoebae. In the present work we found a second *sams* (*sams2*) gene of amoebae, encoding 390 amino acids. Results of localizing X-bacteria SAMS in xD amoebae and measuring SAMS activities and SAM amounts in D and xD amoebae show that the half SAMS activity found in xD amoebae comes from SAMS2 of amoebae and not from their endosymbionts. Interestingly, the expression of two amoeba *sams* genes was switched from *sams 1* to *sams2* by infection with X-bacteria. This is the first report showing the switch in the expression of host *sams* genes by infecting bacteria.

CHAPTER 2
INTRODUCTION

The xD strain of *Amoeba proteus* arose from the D strain by spontaneous infection of X-bacteria (Jeon and Lorch 1967), and xD amoebae are now dependent on their symbionts for survival. Each xD amoeba contains about 42,000 symbionts within symbiosomes, and established xD amoebae die if their symbionts are removed. Newly infected xD amoebae become dependent on X-bacteria within 18 months (about 200 cell generations) (Jeon and Ahn 1978), but the mechanism for the development of host dependence on their symbionts remains unknown. X-Bacteria resemble *Legionella* sp. in their nucleotide sequences of *GroEL* genes (Ahn, 1994) and those of rRNA genes (K. J. Kim, unpublished).

Earlier, we had found that symbiont-bearing xD amoebae did not transcribe the *sams* gene (*sams1*) encoding S-adenosylmethionine synthetase (SAMS) and no longer produced their own SAMS enzyme (Jeon and Jeon 2003; Choi et al. 1997; Ahn and Jeon 1983). However, xD amoebae still showed about half the level of SAMS activity found in symbiont-free D amoebae, despite their inability to express *sams*. Thus, it appeared that symbiotic X-bacteria suppressed the expression of amoeba's *sams* and in turn provided the enzyme for their hosts, enforcing host amoebae to become dependent on symbionts themselves.

The SAMS enzyme catalyzes the formation of S-adenosylmethionine (SAM) from methionine and ATP (Thomas and Surdin-Kerjan 1997) and is essential for cellular survival. SAM is the major methyl donor and precursor for the biosynthesis of polyamines, spermidine, and the phytohormone ethylene. In addition, it is an intracellular signal that controls essential cellular functions such as cell growth and differentiation in both eukaryotic and prokaryotic organisms (Kim et al. 2003; Mato et al. 2002; Shen et al. 2002; Thomas and Surdin-Kerjan 1997). Therefore, lack of SAMS would be detrimental to cells.

In this study, we set out to determine if symbionts supplied SAMS or SAM to the host xD amoebae after inactivating amoeba's own SAM production, by localizing X-

bacteria SAMS in xD amoebae and by measuring SAMS activities in D and xD amoebae. We found that the expression of the normal amoeba *sams* gene (*sams1)* was switched to that of *sams2* by endosymbiotic X-bacteria. Here, we present complete nucleotide and amino-acid sequences of the second amoeba *sams* gene (*sams2*) and its product. Results of our study show that the SAMS activity detected in the xD amoeba cytosol comes from an amoeba SAMS isoform and not from the symbionts. This is the first report on switching of *sams* genes brought about by endosymbionts.

CHAPTER 3
MATERIALS AND METHODS

Amoebae. The D and xD strains of *A. proteus* were cultured in a modified Chalkley's solution (Jeon and Jeon 1975) in Pyrex baking dishes (35 × 22 × 4 cm). Amoebae were fed every other day with axenically cultured and washed *Tetrahymena pyriformis* as food organisms (Goldstein and Ko 1976). The growth rates of amoebae were examined by growing individual amoebae in Syracuse watch glasses in Chalkley's medium containing different concentrations of SAM and by counting the number of amoebae at intervals. SAM, in a stable form of sulfate-p-toluenosulfonate salt produced by Delta Pharmaceutical (Milano, Italy), was kindly provided by Dr. Giovanni Frare. The medium and food organisms were renewed every other day, and control groups were kept in Chalkley's solution without SAM. When desired, we removed X-bacteria from xD amoebae by growing them at 27 °C (Jeon and Ahn 1978).

Uptake of SAM into amoebae. S-adenosyl-L-[*methyl*-^{3}H]methionine (0.55 mCi/ml, 66.8 Ci/mmol) was purchased from ICN Biomedicals (Irvine, CA). Amoebae were prepared to a density of 2.0×10^3 amoebae/ml in Chalkley's solution, and S-adenosyl-L-[*methyl*-^{3}H]methionine was added to a final concentration of 2 μCi/ml. After adding radiolabeled-SAM into media, 1 ml of amoeba culture was transferred into 1.5 ml microcentrifuge tubes at intervals followed by immediate centrifugation for 1 min at 2000 × g. The pelleted amoebae were used in measuring total amount of SAM incorporated into cells, and an aliquot of the supernatant in calculating remained SAM in the medium. The amoebae were resuspended in 150 μl of 0.5-M $HClO_4$ and then loaded on top of 2.5-cm Whatman P-81 phosphocellulose paper discs (Choi et al., 1997). The radioactivity was measured in a Beckman scintillation spectrometer. Net transport and incorporation of SAM into macromolecules was estimated after separating intracellular and incorporated SAM into macromolecules by sonicating the harvested amoebae in 150 μl of 0.5-M $HClO_4$ for 15 sec following centrifugation for 5 min at 12,000 × g.

Recombinant proteins and generation of antibodies against X-bacteria SAMS. X-Bacteria SAMS was produced as a fusion protein in *E. coli* BL21 (DE3) transformed with *xbsams*/pET30a, constructed by inserting 719-bp fragments (nt 367 -- 1085 from the translation-initiation codon) of the coding sequence of X-bacteria *sams* (Jeon and Jeon 2003) into *Bam*HI/*Eco*RI sites of a pET30a vector. The fusion protein contained a His-tag stretch sequence and was purified by metal-affinity chromatography on His-bind Resin (Novagen) in the presence of 6-M urea. We obtained 719-bp fragments by amplifying X-bacteria DNA with two PCR primers, a 5′-primer corresponding to nt 367 -- 380 (5′-CCCGGATCCATGTTTGGTTATGC-3′), and a 3′-primer nt 1,085 -- 1,072 (5′-CCCGAATTCCCATAATGTCCATA-3′) of X-bacteria *sams* genes, and by digesting them with *Bam*HI and *Eco*RI. A *Bam*H1 restriction site was incorporated into the 5′-primer and an *Eco*RI site into the 3′-primer.

An anti-SAMS mouse antibody against a recombinant X-bacteria SAMS was generated by inoculating purified SAMS into mice as described by Jeon and Jeon (2003), and it was used in immunofluorescence microscopy and Western blotting.

Indirect immunofluorescence microscopy. Indirect immunofluorescence microscopy was performed as described by Oh and Jeon (1998). Briefly, about 100 amoebae placed on a glass slide were quickly frozen on dry ice in a drop of 45% acetic acid under a siliconized cover glass. The frozen cells were permeabilized in cold methanol (-20 ˚C) for 5 min. After washing with PBS, amoebae were successively incubated with polyclonal antibodies (pAbs) against SAMS of X-bacteria or amoebae (Jeon and Jeon 2003) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 1 hr each in a moist chamber at room temperature. Slides were thoroughly washed in PBS after each incubation. Then, stained amoebae were mounted in a solution containing 90% glycerol, 1 mg/ml p-phenylenediamine and 10% PBS (Platt and Michael 1983), and examined under a Leitz epifluorescence microscope.

Preparation of protein extracts and assay of SAMS activities. We extracted whole-cell proteins of amoebae by briefly sonicating them in 3 vol. of an extraction buffer (100-mM Tris-HCl, pH 8.0, 5-mM DTT, 50-μM PMSF) and by centrifuging for 10 minutes at 13,000 *g*. The amount of proteins in the supernatant was determined by the Bradford method (Bradford 1976), and 50 -- 100 μg of proteins were used in the assay for the SAMS activity as described previously (Choi et al. 1997). X-Bacteria inside xD amoebae were not broken by brief sonication and were precipitated by subsequent centrifugation. Therefore, SAMS activity of the supernatant obtained from xD amoebae represented that of host amoebae alone not including symbionts' SAMS.

Determination of SAM level. SAM levels were determined as described by Shapiro and Ehninger (1966). Amoebae (50 -- 100 mg) were deproteinized by intermittently shaking with 2 vol. of 1.5-M $HClO_4$ for 1 hr. The extract was neutralized with 3-M $KHCO_3$ and centrifuged for 20 min at 10,000 *g*. The supernatant was incubated with 300 μl of Dowex 50W-X8 resin (equilibrated in 0.1-M NaCl) for 30 min with agitation. The resin was washed with 0.1-M NaCl until the OD_{260} was below 0.05. SAM was eluted from the resin with 5 ml of 6-N H_2SO_4, and its concentration was determined by measuring OD_{256} (ε = 15,400). The amount of SAM in xD amoebae represented the total SAM including that of X-bacteria in xD amoebae, since X-bacteria were completely disrupted by treatment with 1.5-M $HClO_4$ for 1 hr during SAM extraction. The amount of X-bacteria SAM was measured in X-bacteria isolated from xD amoebae. X-Bacteria were isolated by a modified method of Kim et al. (1994). In brief, xD amoebae were suspended in 5 vol. of 20-mM Tris buffer (pH 7.4) by vigorous vortexing, sonicated for 10 sec, and centrifuged for 5 min at 170 *g*. The pellet was resuspended in the same buffer, sonicated again, and centrifuged for 5 min at 170 *g*. The first and second supernatant fractions were combined and centrifuged for 5 min at 7,000 *g*. The pellet after washing with the same buffer was used in measuring SAM level for X-bacteria.

Fractionation of SAMS by Sephadex G-150 and DEAE-cellulose chromatography. SAMS was obtained by chromatography performed after Liau et al. (1977). Cytosol

extract of amoebae was prepared by homogenizing them in 3 vol. of 0.01-M Tris-HCl (pH 7), 5-mM $MgCl_2$, 0.2-mM $CaCl_2$, and 10-mM DTT in a glass homogenizer. The resulting suspension was successively centrifuged at 900 *g* for 10 min and 226,000 *g* for 1 hr to obtain the cytosol extract. The cytosol extract (3 ml) was applied to a column (2.7 × 95 cm) of Sephadex G-150 equilibrated with 0.05-M Tris-HCl (pH 7.8), 50-mM KCl, 5-mM $MgCl_2$, and 20% glycerol. The same solution was used to elute the enzyme at a flow rate of 2.7 ml/fraction/15 min. The OD_{280} profile was obtained for each fraction, and the SAMS profile was determined from a 25-µl aliquot from each fraction assayed for the enzyme activity. The Sephadex G-150 column was previously calibrated with the following molecular markers; catalase (232 kDa), aldolase (158 kDa), and albumin (67 kDa). Blue dextran was used to determine the void volume (V_0).

The DEAE-cellulose was washed with 2-M NaCl and water, and it was suspended in the same buffer used in Sephadex G-150 chromatography. The cytosol extract (3 ml) was applied to a DEAE-cellulose column (2.7 × 8 cm), and the column was washed with the same solution until unadsorbed proteins were washed off the column. The enzyme was then eluted with 150 ml of a linear gradient of 50-mM KCl /5-mM $MgCl_2$ to 500-mM KCl /50-mM $MgCl_2$ in 0.05-M Tris-HCl (pH 7.8) and 20% glycerol. The OD_{280} and SAMS profiles were obtained as described above. The active fractions were analyzed by Western blotting using pAbs against X-bacteria SAMS or amoeba SAMS (Jeon and Jeon 2003).

Cloning the amoeba *sams2* gene. On the basis of a homology alignment of several amino acid sequences of SAMS (Jeon and Jeon 2003; Choi et al. 1997; Yocum et al. 1996), we designed a pair of degenerate PCR primers corresponding to highly conserved amino acid sequences of known SAMS homologs. The 5′- primer corresponded to nucleotides for amino acids 127 to 132 of the amoeba SAMS, Gly-Ala-Gly-Asp-Gln-Gly (5′-CCCGGATCCGGNGCNGGNGAYCARGG-3′), and the 3′-primer was for residues 308 to 314, Gly-Ala-Phe-Ser-Gly-Lys-Asp (5′-CCCGAATTCRTCYTTNCYNGARAANBHNCC-3′), of the same SAMS (Jeon and Jeon 2003). The primers introduced a *Bam*HI site at the 5′-end and an *Eco*RI site at the

3′-end of amplified fragments. PCR was performed under low-stringency annealing conditions (50 ℃ for 1 min) with xD amoeba cDNAs as templates. The cDNAs of xD amoebae were synthesized by reverse transcription with MMLV reverse transcriptase using oligo(dT) primers and mRNAs of xD amoebae, which had been extracted by SV Total RNA Isolation System and PolyATtract mRNA Isolation System III (Promega). The amplified 500-bp fragments were cloned into pBSKII$^+$ vectors and then sequenced. We obtained a 500-bp stretch of the amoeba *sams* gene, which was different from amoeba *sams* previously reported (Choi et al. 1997), and used it in extending 5′- and 3′-end sequences.

The 5′- and 3′-end sequences of *sams2* were obtained by Rapid Amplification of cDNA Ends (RACE) described by Frohman (1990). For 3′-RACE analysis, cDNAs of xD amoebae, which were synthesized with oligo(dT) primers, were amplified with oligo(dT)-anchor primers (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTT-3′) and *sams2*-specific forward primers (SAM1F) corresponding to the nucleotides at nt 435 -- 452. First round PCR products were reamplified with the adapter primers (5′-GACTCGAGTCGACATCG-3′) and SAM2F primers, located at nt 715 -- 732. The second-round PCR products were subcloned into a pGEM-T easy vector (Promega) and sequenced. For 5′-RACE analysis, cDNAs were synthesized with a gene-specific SAM2R primer, followed by attaching poly-A tail to cDNAs with dATP and terminal deoxynucleotidyl transferase (Promega). The tailed cDNAs were amplified with oligo(dT)-anchor primers and gene-specific SAM2R primers. First-round PCR products were reamplified with adapter primers and SAM1R primers. The SAM1R and 2R primers were located at the same positions as SAM1R and 2F primers, respectively, but in reverse sequences. The second-round PCR products were subcloned and sequenced.

Northern blot analysis. To determine the amounts of amoeba *sams1* and *2* transcripts, we introduced isolated X-bacteria into D amoebae (Kim et al. 1994) and prepared total RNA using the SV Total RNA Isolation System (Promega) from samples collected every 4 days for 4 weeks after infection as described by Jeon and Jeon (2003)). We carried out Northern-blot analysis by the usual method (Ausubel et al. 1992). For probing amoeba

sams1 and *2* mRNAs on Northern blots, 1.1-kb fragments (nt 106 -- 1,212) of *sams1* and 0.9-kb fragments (nt 1 -- 910) of *sams2*, respectively, amplified by PCR were labeled with [^{32}P]-dCTP by the Prime-a-Gene labeling system (Promega). For probing *mysosin* mRNA, 0.5-kb fragments (nt 989 -- 1455) of amoeba *myosin* (Oh and Jeon 1998) were amplified and labeled with [^{32}P]-dCTP.

CHAPTER 4
RESULTS

Growth rates of amoebae with added SAM. In order to test the hypothesis that the xD amoeba's dependence on its symbionts for survival is related to the compensation of SAMS or SAM by symbionts, we first determined if host amoebae could survive in media supplemented with SAM following the removal of X-bacteria. We grew xD amoebae for 12 days at 27 ˚C to remove X-bacteria. At room temperature (Fig. 1A), there was no difference in growth rates between D and xD amoebae cultured in SAM concentrations of 10 and 50 μM. However, at 100 μM, D amoebae died within 10 days, and xD amoebae within 4 days. The results implied that SAM at 100 μM was harmful to amoebae, and that xD amoebae were more sensitive to a high concentration of SAM than were D amoebae.

At 27 ˚C (Fig. 1B), all xD amoebae died within 2 weeks as was previously known. In contrast, D amoebae grew well even in 10-μM SAM but died after 11 days at concentrations of 50- and 100-μM SAM. The results indicated that D amoebae became sensitive to a high temperature in 50 μM SAM or higher and that xD amoebae could not survive after the removal of X-bacteria even when SAM was supplied in culture media. However, the results did not exclude the possibility that X-bacteria provided SAMS or SAM to their host amoebae, since even D amoebae became sensitive to high temperatures in the presence of SAM.

Uptake of SAM into amoebae. Both D and xD amoebae showed same pattern of time-dependent uptake of SAM, but D amoebae showed only approximately 36% uptake of SAM found in xD amoebae (Fig. 2). To check the ratio of intracellular transported and membrane or some structure-incorporated SAM, we broke radiolabeled amoebae by brief sonication in 0.5-M $HClO_4$ and then separated soluble cellular cytosols and insoluble pellets by centrifugation. The total uptake consisted of two distinguishable components, incorporation of SAM into insoluble macromolecules (75%) and transport of SAM into

amoebae (25%) in D and xD amoebae. Incorporation of SAM was linear continually, but net transport was linear for the first 20 min and the rate declined thereafter.

Localization of X-bacteria SAMS. In order to determine if X-bacteria provided SAMS to their hosts, we localized X-bacteria SAMS by indirect immunofluorescence microscopy using polyclonal antibodies (pAb) against X-bacteria or amoeba SAMS. When D amoebae were stained with pAb against X-bacteria SAMS, antigens were found dispersed throughout the cytoplasm (Fig. 3A). In contrast, only symbiosomes were heavily stained and no antigen was detected in the cytoplasm of xD amoebae. The results indicated that X-bacteria SAMS was present only within symbiosomes of xD amoebae and that it was not transported to the cytoplasm of amoebae. In Western blotting using total proteins of D or xD amoebae, pAb against X-bacteria SAMS or amoeba SAMS showed cross-reactivity with amoeba SAMS or X-bacteria SAMS (Data not shown). Same results were obtained when amoebae were stained with pAb against D-amoeba SAMS (Fig. 3B).

Activity of SAMS. In order to confirm that X-bacteria did not provide SAMS to their hosts as shown by the above localization experiments using pAb against X-bacteria or amoeba SAMS, we measured SAMS activities of D and xD amoebae. xD Amoebae grown at room temperature had about 57% of SAMS activity found in D amoebae (Fig. 4).

For excluding any potential contamination by X-bacteria SAMS, we measured SAMS activities in symbiont-free xD amoebae grown for 8 days at 27 °C. Even though X-bacteria were completely removed from xD amoebae by growing at 27 °C, as checked under a phase-contrast microscope, xD amoebae showed the same level of SAMS activities as at room temperature. Together with the data obtained from the localization experiments using pAb against X-bacteria SAMS, these results indicated that the SAMS activity found in xD amoebae, which was about half that of D amoebae, came from host amoebae and not from X-bacteria.

Characterization of SAMS from D and xD amoebae. The above results suggested that there might be a SAMS isoform in amoebae, having a different amino-acid sequence from that of the previously studied amoeba SAMS. In order to check if there was such an isoform, we fractionated proteins of D and xD amoebae in a Sephadex G-150 or DEAE-cellulose column and then identified SAMS by Western blotting using pAb against amoeba SAMS1 and X-bacteria SAMS. The elution patterns of SAMS from D and xD amoebae on Sephadex G-150 are shown in Fig. 5A. Three peaks with SAMS activity were detected after gel filtration of the D-amoeba cytosol extract. These peaks coincided with the regions with molecular masses corresponding to 210, 160, and 100 kDa, respectively. In contrast, most xD SAMS was eluted in two regions of 210 and 100 kDa, respectively.

Figure 5B illustrates the elution patterns of SAMS from D and xD amoebae in DEAE-cellulose columns, showing that enzyme profiles of D and xD amoebae were different. While xD amoeba SAMS was eluted as a sharp single peak at 0.2-M KCl, D amoeba SAMS was eluted as a broad major peak between 0.2- and 0.25-M KCl with a shoulder extending to 0.5-M KCl.

For checking if amoeba SAMS1, amoeba SAMS previously found only in D amoebae, was contained in the regions with peak activities, aliquots of fractions around the peak activity shown in Fig. 5 were subjected to SDS-PAGE and immunoblotting with pAb against amoeba SAMS1. Western blotting showed a single band of 45 kDa when aliquots of D amoebae were analyzed. In contrast, no band was detected with the same pAb when aliquots of xD amoeba proteins were analyzed (Fig. 6). The results indicated that the SAMS activity detected in xD-amoeba proteins did not come from amoeba SAMS1.

Figure 6 shows the results of Western blotting for the aliquots obtained by gel filtration. When pAb against X-bacteria SAMS were used, or when aliquots obtained by DEAE-cellulose chromatography were analyzed by Western blotting, the same results were obtained (Data not shown). These results indicated that there was another amoeba SAMS (SAMS2) that was different from the previously reported SAMS1.

Complete nucleotide and deduced amino-acid sequences of *sams2*. In order to clone the second *sams* gene (*sams2*) from amoebae, we reverse transcribed and amplified mRNAs of xD amoebae. We obtained a 1.2-kb fragment of *sams2* by extending both ends of a 500-bp stretch of *sams2* that had come from an amplified xD-amoeba cDNA. We used degenerate PCR primers corresponding to DNA sequences encoding amino acids that were highly conserved among known SAMS (Choi et al. 1997; Yocum et al. 1996).

The *sams2* gene had an ORF of 1,173 nt (Fig. 7), encoding 390 amino acids, and it was similar to that of other organisms. The SAMS2 protein had a mass of 43 kDa, with pI 6.1, as determined by ExPASy Molecular Biology server.

Comparison of deduced amino-acid sequences of SAMS2 with other SAMS homologs. Multiple sequence alignment of amoeba SAMS2 with other SAMS homologs (Fig. 8) revealed that SAMS2 contained a consensus ATP-binding motif (GAGDQG at position 124 -- 129) (cf. Takusagawa et al. 1996), the glycine-rich nanopeptide (GGGAFSGKD at position 271 -- 279) (Jeon and Jeon 2003; Choi et al. 1997), and metal-binding sites (Asp-24, Asp-285, and Glu-50) (cf. Reguera et al. 2002; McQueney and Markham 1995). The X-bacteria SAMS and amoeba SAMS2 had a glycine residue at position 121, whereas SAMS1 had valine at the position. The sequence identity of SAMS2 with SAMS1 of *A. proteus* or that of X-bacteria was 50%, while it was 72% with *Acanthamoeba castellanii*, 66% with *Phytophthora infestans*, and 52% with *E. coli*.

Northern blot analyses of *sams1* and *sams2* gene expression after infection. In order to ensure that the DNA sequence of *sams2* gene was that of amoebae and not of X-bacteria, we analyzed genomic DNAs of D and xD amoebae and of X-bacteria by Southern blotting using a *sams2* probe after digesting DNAs with *Eco*RI. We found a common band in DNAs of D and xD amoebae but not in X-bacteria DNA (data not shown).

In order to examine the expression profiles of *sams1* and *sams2* in newly infected xD amoebae, we infected D amoebae with X-bacteria and determined the amount of *sams* mRNAs at intervals by Northern blotting (Fig. 9). In agreement with our previous findings (Jeon and Jeon 2003), the amount of *sams1* transcript decreased to a negligible level within 4 weeks following infection. In contrast, the amount of *sams2* transcript slightly increased after infection with X-bacteria.

The amount of SAM. The above results suggested a possible switching between the two *sams* genes in amoebae as a result of symbiosis with X-bacteria. To study the consequence of such switching, we measured the size of intracellular pools of SAM in the two amoeba strains and X-bacteria (Fig. 10). At room temperature, D amoebae showed 0.614 nmol/mg of SAM. Surprisingly, xD amoebae contained a similar amount of SAM (0.621 nmol/mg), which was obtained by subtracting the amount of X-bacteria SAM from that of xD amoebae containing X-bacteria.

In order to test if X-bacteria provided SAM to their host amoebae without contributing SAMS, we measured the SAM level after removing X-bacteria by growing xD amoebae for 8 days at 27 ˚C. Even after X-bacteria in xD amoebae had been removed, the levels of SAM in D and xD amoebae remained similar, indicating that X-bacteria did not provide SAM to their hosts.

CHAPTER 5
DISCUSSION

The most significant finding from our study is that amoebae have a second *sams* gene (*sams2)* that is transcriptionally activated by symbiotic X-bacteria, while endosymbionts suppress the expression of *sams1*, the normally transcribed gene in symbiont-free amoebae. This is the first report showing such a switch in the expression of host *sams* genes occurring as a result of symbiosis.

In previous studies, we found that symbiont-bearing xD amoebae did not transcribe the *sams* gene and no longer produced their own SAMS as a result of harboring X-bacteria (Jeon and Jeon 2003; Choi et al. 1997; Ahn and Jeon 1983). Yet, xD amoebae still showed about half the level of SAMS activity found in symbiont-free D amoebae. It appeared that X-bacteria suppressed the expression of amoeba's *sams* and in turn provided the enzyme for their hosts. Thus, it was suspected that the lack of SAMS in xD amoebae was compensated by their bacterial endosymbionts and that such compensation might be the reason for amoebae to become dependent on their symbionts for survival. Our present study was aimed at elucidating the mechanism for such gene suppression. Unexpectedly, our results show that the SAMS activity found in xD amoebae comes from the second amoeba SAMS (SAMS2) and not from the endosymbionts. It appears that the reason why no SAMS was detected in xD amoebae in previous studies by immunoblotting using a pAb against amoeba-SAMS1 was because SAMS2 of amoebae had only 50% identity in amino-acid sequence with amoeba SAMS1 and thus it did not react with the pAb. Measurements of the SAMS activity and the intracellular level of SAM in amoebae confirm that X-bacteria do not provide SAMS or SAM to their hosts. The intracellular level of xD amoeba SAM is similar to that of D amoebae even though xD amoebae possess only about half the activity of SAMS compared with D amoebae. The fact that X-bacteria SAMS is localized only in symbiosomes of xD amoebae supports the above view.

It is known that the expression of *sams* genes is switched from *MAT1A* to *MAT2A* in rat and human-derived hepatoma cell lines and human hepatocellular carcinoma

(HCC), which facilitates cancer cell growth (Avila et al. 2000; Cai et al. 1998; Cai et al. 1996). Similar results were also found after partial hepatectomy (Huang et al. 1998) and in human liver cirrhosis (Avila et al. 2000). Human methionine adenosyltransferase (MAT) is encoded by two genes, *MAT1A* (liver-specific) and *MAT2A* (non-liver-specific). Differential expression of individual *sams* genes during growth and development has been reported in mammals (Mato et al. 2002), *Catharanthus roseus* (Schroder et al. 1997), and *Pisum sativum* (Gomez-Gomez and Carrasco 1998).

Enteric bacteria are not permeable to SAM (Sekowska et al. 2000), but *S. cerevisiae* is capable of actively transporting SAM (Thomas and Surdin-Kerjan 1997). It is not clear if mammalian cells take up exogenous SAM and conflicting data are found in the literature (Watson et al. 1999; Bontemps and Van Den Berghe 1997). Meanwhile, intracellular levels of SAM in isolated rat hepatocytes increase when external SAM concentration is 200 μM or higher (Watson et al. 1999). Incubation of isolated rat hepatocytes with 2 or 50 μM of SAM causes the methyl group of exogenous SAM to be incorporated, without intracellular uptake, into phospholipids most probably situated on the outside of the plasma membrane, forming phosphatidylcholine (Bontemps and Van Den Berghe 1997).

It is interesting that amoebae are permeable to SAM in the medium, and that most of the exogenous SAM taken up is incorporated into precipitable cell components without concomitant intracellular uptake (cf. Fig. 2). In our study, both D and xD amoebae showed time-dependent uptake of SAM, but xD amoebae showed much higher uptake rate of exogenous SAM found in D amoebae. It is not clearly known as to which cell components incorporate exogenous SAM, but it is likely that the radiolabeled methyl group of SAM is incorporated into phospholipids of the plasma membrane. It also appears that the temperature sensitivity of amoebae in the presence of SAM is due to the change in the fluidity of amoeba membrane caused by exogenous SAM. xD Amoebae cannot survive at 27 ˚C or above and all die within 2 weeks (Jeon and Ahn 1978) because symbiotic bacteria disappear apparently by digestion (Lorch and Jeon 1980). The higher uptake rate of exogenous SAM by xD amoebae appears to be the reason for xD amoeba's higher sensitivity to SAM since a high concentration of SAM is harmful to amoebae.

However, little is known about the role of SAM in amoebae and in the amoeba /X-bacteria symbiosis. xD Amoebae are more sensitive to exogenous SAM than are D amoebae as shown by their lower growth rates in the presence of SAM. However, it is not known why xD amoebae are more sensitive to exogenous SAM and why amoebae become sensitive to an elevated temperature in the presence of SAM. It may be related to the general fragility of their membranes since it is known that xD amoebae are more sensitive to overfeeding, starvation, micrurgical operations, and elevated culture temperature (Jeon 1995).

Multiple sequence alignment of SAMS2 with other SAMS homologs reveals that SAMS2 also contains a consensus ATP-binding motif (GAGDQG) and the glycine-rich nanopeptide (GGGAFSGKD). The SAMS enzyme catalyzes the only known biosynthetic route to SAM, the primary biological methyl donor (McQueney et al. 2000). SAM also acts as an intracellular signal that controls essential cellular functions such as cell growth and differentiation as well as sensitivity to liver injury (Kim et al. 2003; Mato et al. 2002). In *Streptomyces*, an elevated level of SAM inhibits sporulation and a certain level of intracellular SAM is critical for the induction of antibiotic biosynthetic genes (Kim et al. 2003; Okamoto et al. 2003). In *E. coli*, a lowered level of SAM increases C to T mutagenesis (Macintyre et al. 2001) and results in a division defect (Newman et al. 1998). The administration of exogenous SAM attenuates liver injury induced by lipopolysaccharide treatment in rats (Watson et al. 1999; Chawla et al. 1998).

Genomic and cDNA clones of *sams* have been obtained from various organisms (Jeon and Jeon 2003; Schroder et al. 1997; Sakata et al. 1993; Horikawa and Tsukada 1991; Peleman et al. 1989; Thomas and Surdin-Kerjan 1987; Markham et al. 1984). Most organisms studied to date have more than one SAMS isozymes. The *sams* genes comprise a small gene family, and they appear to be highly conserved at least within a species (Mato et al. 2002; Shen et al. 2002; Reguera et al. 2002; Thomas and Surdin-Kerjan 1997).

Amoeba's SAMS2 enzyme we identified has only 50% amino-acid sequence identity with SAMS1 of amoebae and X-bacteria SAMS. The sequence identity between the two isoforms in amoebae is unusually low since all other known SAMS isoforms of

an organism show over 80% sequence identity. Based on the sizes of proteins determined by gel filtration, there seem to be three different conformations of D amoeba SAMS, while there seem to be only two different conformations of SAMS in xD amoebae. However, the reason why amoeba SAMS has different sizes is not clear.

In amoebae, *sams2* is expressed at a low level in symbiont-free D amoebae but it is activated by X-bacteria. xD Amoebae contain a similar amount of SAM as do D amoebae, whereas they have only half the activity of SAMS found in D amoebae. The amount of SAM in xD amoebae measured after the removal of X-bacteria is similar to that of D amoebae, the results suggesting that X-bacteria did not provide SAM to their host amoebae. It is interesting that xD amoebae show only half the activity of SAMS while containing a similar amount of SAM as do D amoebae. The discrepancy between SAMS activity and the amount of SAM in xD amoebae might be due primarily to a lower estimate of SAMS activity in xD amoebae. Possible difference in K_m values for the enzyme substrates present in the two amoeba strains or different V_{max} of the enzymes might have caused lower estimation of SAMS activity in xD amoebae. In our study, we used constant concentrations of substrates, methionine and ATP for measuring the activity of SAMS. Different properties of SAMS isozymes have been reported in mammals (Mato et al. 2002) and in yeast (Thomas and Surdin-Kerjan 1997).

It is also possible that activities of enzymes consuming SAM such as glycine-N-methyltransferase (GNMT) might be higher in D amoebae or lower in xD amoebae. The main function of GNMT is to remove excess SAM synthesized during a methionine load (Mato et al. 2002). When large quantities of SAM are synthesized by the liver, the excess SAM is not used to hypermethylate DNA and/or proteins or for the synthesis of polyamines, which would be harmful to the liver. Rather, SAM is converted rapidly into S-adenosylhomocysteine by GNMT (Mudd et al. 2001).

It is known that a high concentration of SAM is harmful to cells and the maintenance of proper SAM levels is crucial for the survival of cells (Kim et al. 2003; Mato et al. 2002). It has been suggested that fluctuations in the hepatic SAM concentration could be a part of the priming events and terminating signals that modulate the liver's regenerative process (Latasa et al. 2001). The hepatic SAM level is

dramatically reduced shortly after a partial hepatectomy, and then subsequently it is restored to normal levels.

Further work is in progress to determine the consequences of the *sams* gene switching in amoeba/X-bacteria symbiosis and the switching mechanism. The switch in gene expression in amoebae is not only an example of genetic alterations caused by host-symbiont interactions but also may serve as a good model to study interactions between hosts and infective agents such *as Mycobacterium, Legionella, Toxoplasma, Salmonella,* and others.

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APPENDIX

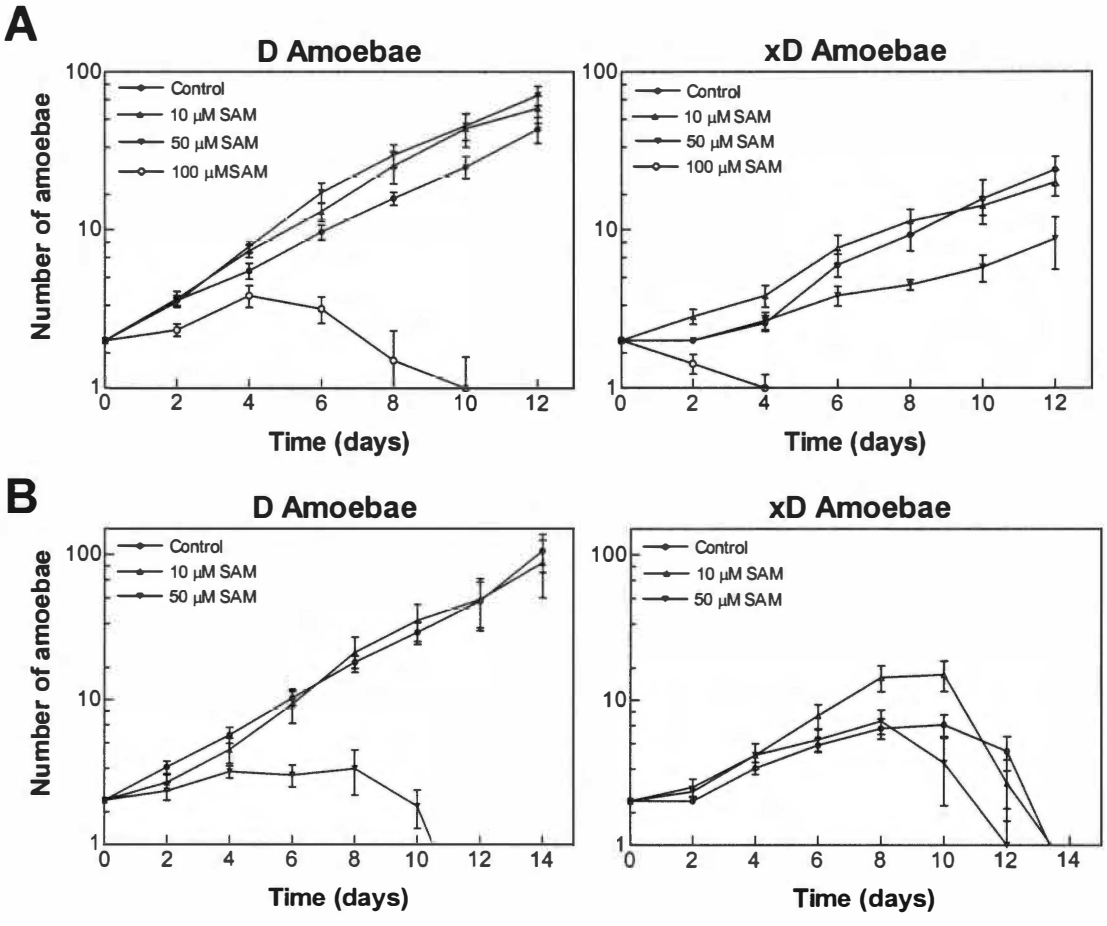

Fig. 1. Growth rates of amoebae at different concentrations of SAM. **A,** growth rates of D and xD amoebae at 22 ˚C; **B,** growth rates at 27 ˚C. Each point represents the average (± SD) from 24 cells grown singly in two different experiments.

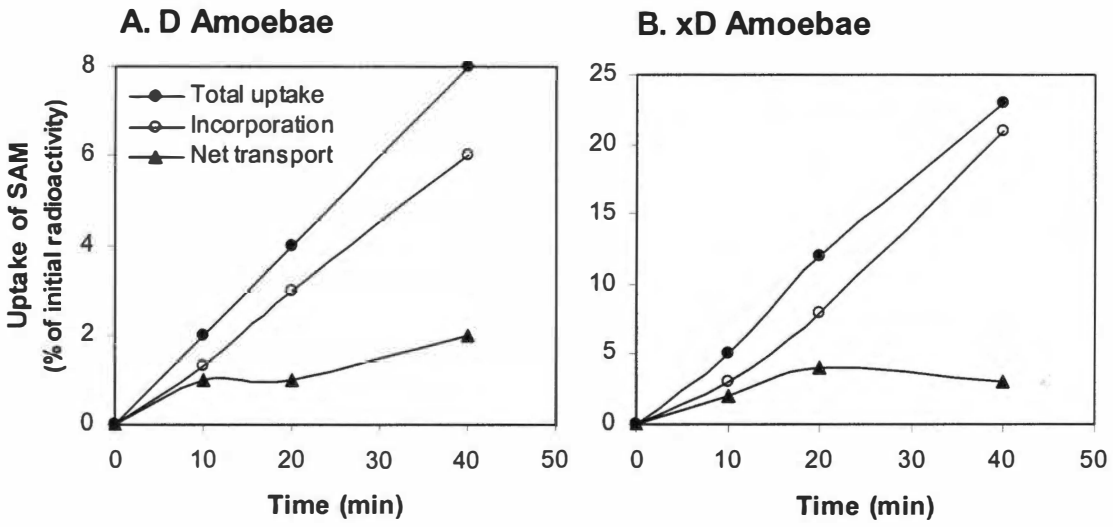

Fig. 2. Uptake of SAM into amoebae. Incorporation rates of SAM into D amoebae (**A**) and xD amoebae (**B**). A density of 2.0×10^3 amoebae/ml in Chalkley's solution was added with S-adenosyl-L-[*methyl*-^{3}H]methionine to be a final concentration of 2 μCi/ml. Total uptake (•) was calculated from the precipitated amoebae before sonication. After sonication followed by centrifugation, incorporated SAM (o) was from the pellets, and net transport (▲) from supernatants. Results are expressed as percentage of the initial radioactivity of SAM. Values are means for two separate experiments.

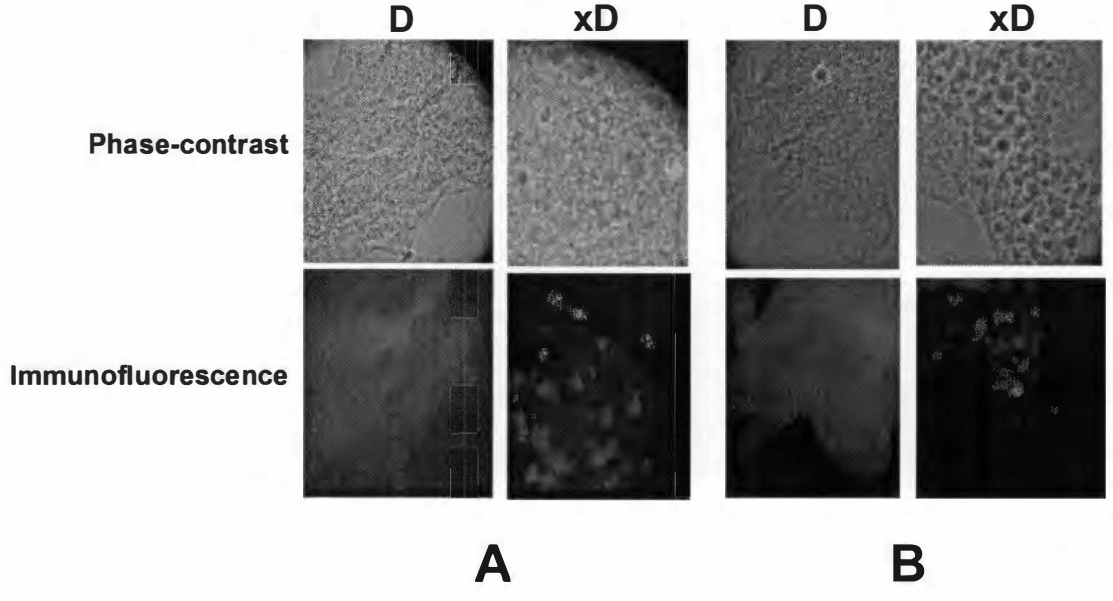

Fig. 3. Localization of X-bacteria SAMS. Indirect Immunofluorescence micrographs of D and xD amoebae stained with an anti-X-bacteria SAMS antibody (**A**) and an anti-amoeba SAMS1 antibody (**B**).

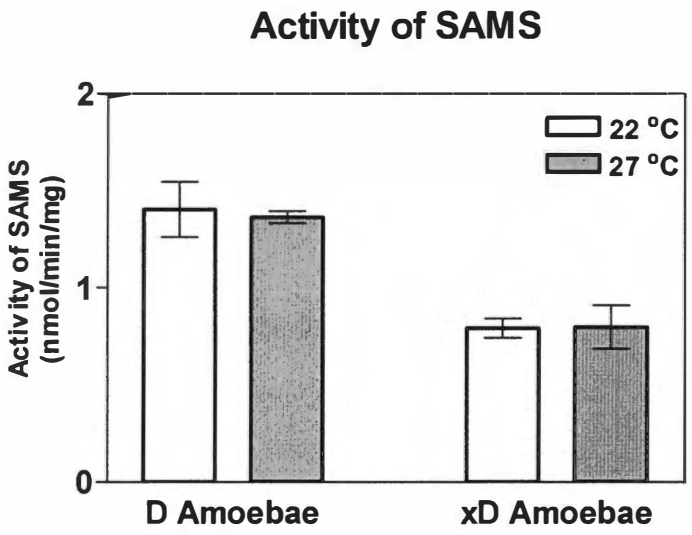

Fig. 4. Graphs to show SAMS activities contained in whole-cell proteins from D and xD amoebae as measured using L-[methyl-H^3]methionine. For removing X-bacteria from xD amoebae, cells were grown for 8 days at 27 °C. The values for xD amoebae represent only the SAMS activities of host amoebae not including symbionts since X-bacteria were excluded during extraction of proteins from xD amoebae. The data represent mean ± SE from three separate experiments.

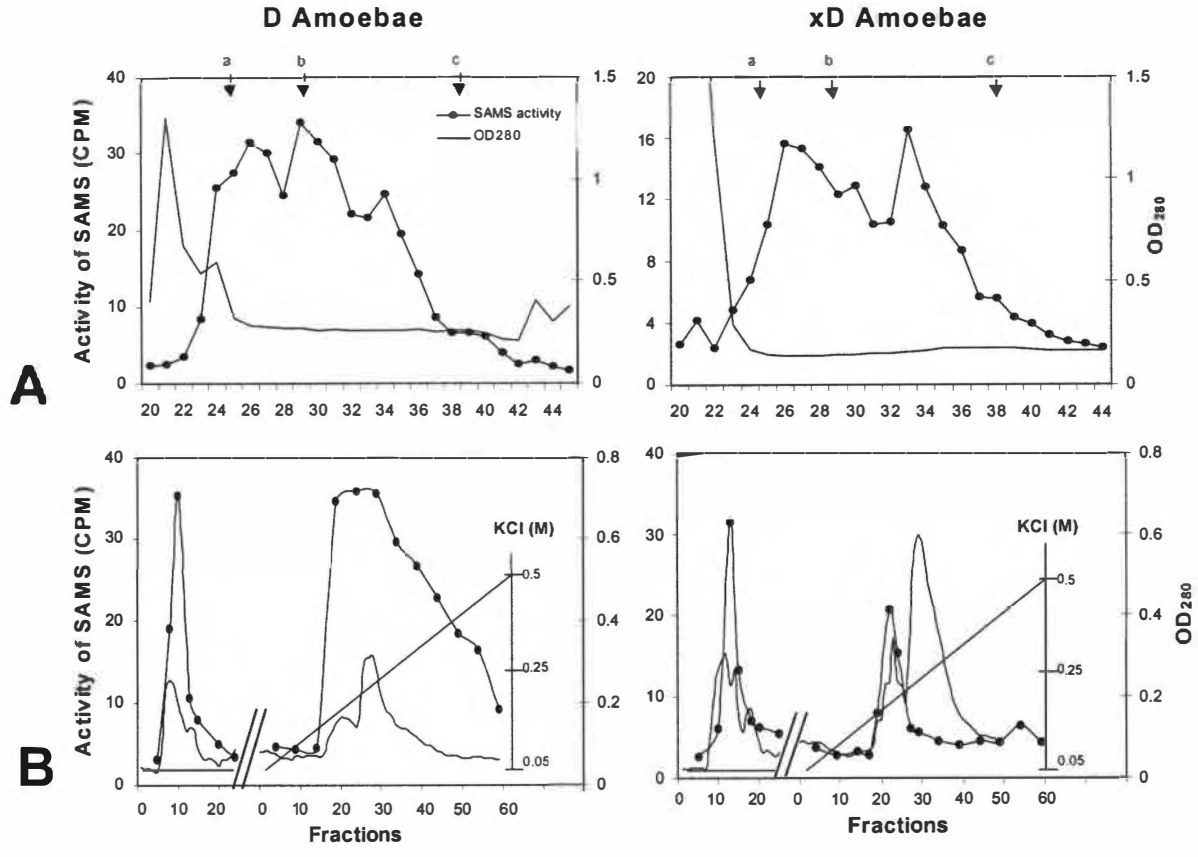

Fig. 5. Fractionation of SAMS from D and xD ameobae. **A**, Results of Sephadex G-150 chromatography of D and xD amoebae, respectively. **B,** Results of DEAE-cellulose chromatography of D and xD amoebae, respectively. Cytosolic extracts (2-3 ml) of D and xD ameobae were chromatographed, and 25-µl aliquot from each fraction was used in the assay of SAMS activity. The Sephadex G-150 column was previously calibrated with the following molecular markers (arrows); a. Catalase (232 kDa), b. Aldolase (158 kDa), and c. Albumin (67 kDa).

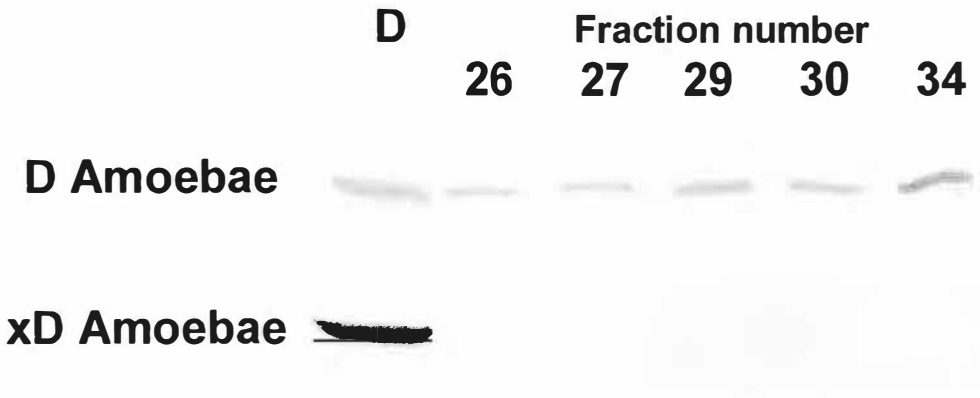

Fig. 6. Western blot analyses of proteins fractionated by Sephadex G-150 chromatography. Fractions containing SAMS enzyme activities (Fig.4) were subjected to Western blotting using pAbs against amoeba-SAMS1. A 15-μl aliquot from each fraction was separated by SDS-PAGE (10%), and transferred to a membrane for Western blotting. Whole-cell proteins of D amoebae were loaded in the first lane as a size marker for amoeba SAMS1.

Fig. 7. Complete nucleotide and deduced amino-acid sequences of *sams2*. The ORF of *sams2* is 1173 nt long and encodes SAMS of 390 amino acids. The start and stop codons are underlined.

```
ATGTCGACTCCTGGTGCACCCCTAGAAACATATTTCTTTACTAGTGAGTGCGTCACTGAAGGCCACCCAGACAAACTGTGTGACCAAGTTTCTGATGCG 99
M  S  T  P  G  A  P  L  E  T  Y  F  F  T  S  E  C  V  T  E  G  H  P  D  K  L  C  D  Q  V  S  D  A   33

GTATTGGATGCGTGCTTGTCAGAGGATCCCTTTTCGAAAGTGGCGTGTGAAACGTCAACCAAGACTGGACTGGTCATGATATTTGGCGAAATCACAACA 198
V  L  D  A  C  L  S  E  D  P  F  S  K  V  A  C  E  T  S  T  K  T  G  L  V  M  I  F  G  E  I  T  T   66

CGTGGTCATCCCGACTACCAGAAAGTTGTGCGGGATGCAGTAAAGCACATTGGATTTGATAATGGCGAAATAGGATTTGACTACAAAACCTGTAATGTT 297
R  G  H  P  D  Y  Q  K  V  V  R  D  A  V  K  H  I  G  F  D  N  G  E  I  G  F  D  Y  K  T  C  N  V   99

ATGGTTTGTATTGAACAACAAAGCCCGGACATTGCTGGTGGGGTACATATTGGTAAGTCCGACGAAGATCTTGGTGCAGGGGATCAAGGCCATATGTTT 396
M  V  C  I  E  Q  Q  S  P  D  I  A  G  G  V  H  I  G  K  S  D  E  D  L  G  A  G  D  Q  G  H  M  F   132

GGATATGCCACGAACGAGACGCCCGAGATGATGCCTTTGACCCATGTCTTGGCCGCTAAATTGGCCAAGGCACTCTCTGATGCCCGAAGATCTGGCTTG 495
G  Y  A  T  N  E  T  P  E  M  M  P  L  T  H  V  L  A  A  K  L  A  K  A  L  S  D  A  R  R  S  G  L   165

GTTCCTTGGCTGCGCCCTGATGCGAAAACACAAGTTACAATCGAATACAAGAATGACCAAGGGAGGGCCGTACCACAACGTGTCCACACAGTTGTAATC 594
V  P  W  L  R  P  D  A  K  T  Q  V  T  I  E  Y  K  N  D  Q  G  R  A  V  P  Q  R  V  H  T  V  V  I   198

TCTGCACAACATTCGCCAGATGTGTCTGTTGAACAGATCCGCAAGGATCTTAAGGAGAAGATCATCATCCCTACCATCCCCGCTCAATATCTGGATGAC 693
S  A  Q  H  S  P  D  V  S  V  E  Q  I  R  K  D  L  K  E  K  I  I  I  P  T  I  P  A  Q  Y  L  D  D   231

CAAACTATTTATCATCTCAATCCATCTGGTAGGTTTGTCATTGGAGGACCACAGGGTGATGCTGGTACAACTGGTCGTAAAATCATCGTGGATACCTAT 792
Q  T  I  Y  H  L  N  P  S  G  R  F  V  I  G  G  P  Q  G  D  A  G  T  T  G  R  K  I  I  V  D  T  Y   264

GGAGGATGGGGAGCTCATGGTGGAGGTGCCTTTTCTGGAAAGGATCCAACCAAGGTTGACCGATCTGCTGCGTATGCATGCCGTTGGATTGCCAAGTCA 891
G  G  W  G  A  H  G  G  G  A  F  S  G  K  D  P  T  K  V  D  R  S  A  A  Y  A  C  R  W  I  A  K  S   297

CTTGTGGCTGCTGGTCTGTGTGATAGATGTCTGGTACAAGTTTCGTATTCTATTGCTGTTGCCCATCCAATGTCGCTCTTCGTTAACTCGTATGGTACT 990
L  V  A  A  G  L  C  D  R  C  L  V  Q  V  S  Y  S  I  A  V  A  H  P  M  S  L  F  V  N  S  Y  G  T   330

GGTAAGAAGTCTGATGCAGAGTTGATGGAGATCATCAAAGCCAACTTCGATTTGCGACCTGGTATGATCGTCCGTGCGCTTAACCTACGTCGACCCATT 1089
G  K  K  S  D  A  E  L  M  E  I  I  K  A  N  F  D  L  R  P  G  M  I  V  R  A  L  N  L  R  R  P  I   363

TATTTCAAGACTGCTTCATTTGGTCATTTTGGACGAGATGACGATGACTTTGAATGGGAGAAACCTAAAAAACTCGTCTTTTAAGTAAAAGCGCAGTAT 1188
Y  F  K  T  A  S  F  G  H  F  G  R  D  D  D  D  F  E  W  E  K  P  K  K  L  V  F  *                390

GATCCTCAAAAAAAAAAAAAAAAAA ---------------------------------------------------------------------- 1213
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Fig. 8. Alignment of deduced amino-acid sequence of amoeba SAMS2 with SAMS sequences of other organisms. Periods represent amino acids identical to those of MetK of *E. coli*, and dashes show gaps inserted for an optimal alignment of amino acids. *1, ATP-binding motif; *2, Glycine-rich nanopeptide; *3, Metal-binding sites; *4, A site for Cys-121 of human *MAT1A* characteristic of liver enzymes. EC, *E. coli*; XB, X-bacteria; AC, *Acanthamoeba castellanii*; S2, SAMS2 of amoebae; PI, *Phytophthora infestans*; AP, *Amoeba proteus*. Complete genomic or cDNA sequences of SAMS proteins are available in GenBank, for *E. coli* (accession number, 1708999), *A. castellanii* (6016547), and *P. infestans* (23394401). The amino-acid sequences of amoeba SAMS1 and X-bacteria SAMS are from Choi et al. (1997) and Jeon and Jeon (2003).

```
                                *3                                  *3
EC     1 ~~~MILNMAKHLFTSESVSEGHPDKIADQISDAVLDAILEQDPK....ARVACETYVKTG
XB     1 ~~~~~~~.II.V.....................I.....AK..L...........F....
AC     1 ~~~~MASSKTL.....C...S....LC..V...I...C.AN..Y....SK.....AS...
PI     1 MTQTTTKDRTF.......N......LC..........CVSE..N....S......CA...
AP     1 ~~~~MQQNTRY..A..A..R.....AC..V..R...YC.QAEK.CKKAS...L..TI.GN
S2     1 MSTPGAPLETYF....C.T......LC..V.......C.SE..F....SK.....ST...
                                                                   *4
EC    54 MVLVGGEITTSAWVDIEEITRNTVREIGYVHSDMGFDANSCAVLSAIGKQSPDINQGVD.
XB    50 ..............V.T...EVIKD...NS......WA..S.........L..A.....
AC    53 ..M.F.....KSSF.YQKVI.E..KR..FTD.SI...YKT.NI.V..EQ.....A...H.
PI    57 ..MIF...S.K.I.NY.KVI.D.IK....DDPAK.L.YKTVN.IV..EQ.....A.S...
AP    57 V.GLF..V.CQKTFPTISWF.EL.T....SRE.LDL.PTT.S.HINVRG.EAE.RGV.HN
S2    57 L.MIF.....RGHP.YQKVV.DA.KH..FDNGEI...YKT.N.MVC.EQ.....AG..H.
                 ******1
EC   113 ..RADPLEQGAGDQGLMFGYATNETDVLMPAPITYAHR....LVQRQAEVRKN.......
XB   109 ..NRETKIL............SR....C.....A.S.......MA....L.........
AC   112 .VGRSDDDL......H...H.....PEF..MTHVL.T......C..MT...N........
PI   116 ..SVTDEDV......I.....SD..PE...LSHVL.TK.....GSKLT............
AP   117 QE..AKETL.............D..PERC.CLWFLLR.FKLA.RSKFE.A..KKIELAVA
S2   116 .IGKSDEDL......H.........PEM..LTHVL.AK.....AKALSDA.RS.......

EC   160 ..............GTLP..WLRPDAKSQVTFQY..DDGKIV..GIDAVVLSTQHSEEI
XB   156 ..............NK.........G.C.L.LK....Q..P...A..TI.F....AP..
AC   160 ..............SI.S...M.....T...VE.RNEN.TLIPLRRHT..I.V....DV
PI   163 ................D...I...G.T...VE.KQE..RM.PQRVHT..I....NDDV
AP   177 KGIKVSQVDATPELRD.IDFW..HT......IIE.ED.S.ALKPITARVA...V...KFV
S2   164 ..............LV..........T...IE.KN.Q.RA.PQRVHT..I.A...PDV

EC   199 DQKSLQEAVMEEIIKPILPAEWL.TSATKFFIN....PTG.RFVIGGPMGDCGLTGRKII
XB   195 SH.D.V...R......V..E....SA..RYY.................L...........
AC   203 TNEEIRKRL...V...SI..HL..DGE.I.HL......S.........Q.EA....G...
PI   206 TNEQIAADL.KHV.QTVI.EKY..DDK.VYHL......S.........H..A........
AP   237 THDQYEDRL.PQLV.GV.DEYGMHSE..EYL..IKQKTS.YGWTV...NA.A.T......
S2   207 SVEQIRKDLK.K..I.TI..QY..DDQ.IYHL......S.........Q..A.T......
                 *********2   *3
EC   253 VDTYGGMARHGGGAFSGKDPSKVDRSAAYAARYVAKNIVAAGLADRCEIQVSYAIGVAEP
XB   249 .............C............G.....H....L....I.EK.............
AC   257 I.....WGA.......................WI..SL..R...N.ALV........SH.
PI   260 I.....WGA..........TT...........W...SV..K...H.LLV.L......PY.
AP   296 ......HGA.....................Y.....Q.L.R.K.CR.VLV....V..KP..
S2   261 ......WGA...........T.........C.WI..SL.....C...LV....S.A..H.

EC   313 TSIMVETFGT..EKVPSEQLTLLVREFFDLRPYGLIQMLDLLHP...IYKETAAYGHFGR
XB   309 ...S.D......GHLRNNVIID.IKTH...T.Q.I.DHH..FS......RQ......Y..
AC   317 L.VF.DSY..AQGGRTD.D.LAISKSN.....GKI.ND.Q.RR......EK..YH.....
PI   320 L..H.DSY..VK.G.TDDD.VEIIKKN.....GMIQKT.Q.KR....VMQK.........
AP   356 LN.Y.N.Y....GTHSDSE.LEIINKN..F..GFI.EE....N.DRIK.V...YH.....
S2   321 M.LF.NSY....G.KSDAE.MEIIKAN.....GMIVRA.N.RR......FK..SF.....

EC   368 E..HFPWEKTDKAQLLRDAAGLK
XB   364 D..GL...RL..VAA.AK.L~~~
AC   374 NDPD.L..APK.LNF~~~~~~~~
PI   377 .DAD.T..TVKELE.~~~~~~~~
AP   414 ..PE....QEKTLT.~~~~~~~~
S2   376 DDDD.E...PK.LVF~~~~~~~~
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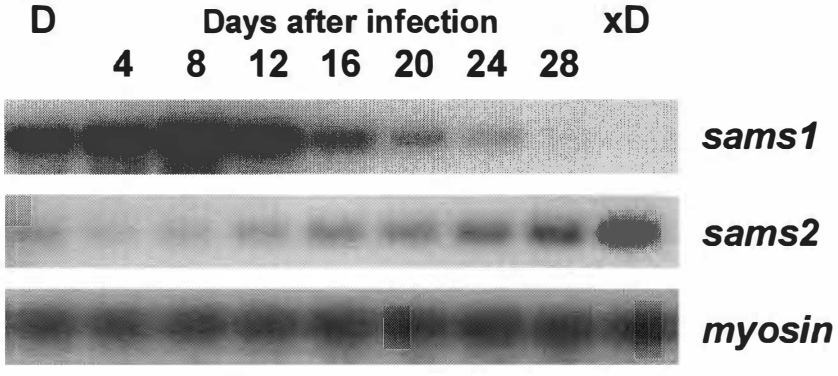

Fig. 9. Northern blot analyses of *sams1* and *sams2* genes following infection with X-bacteria. Total RNAs (20 μg) isolated from amoebae collected every 4 days after infection were separated by formamide gels and then transferred to nylon membranes. The signal was detected subsequently on the same blot with ^{32}P-labeled *sams1*, *sams2*, and *myosin* probes. A *myosin* probe was used as a loading control.

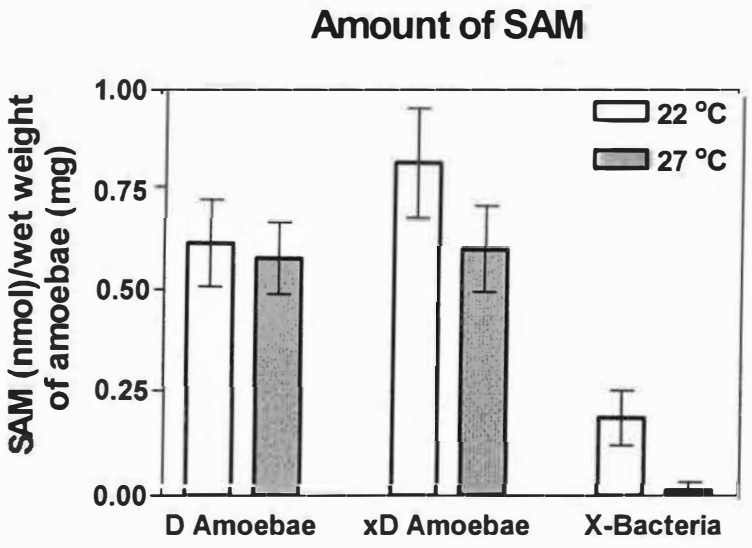

Fig. 10. Graphs to show the size of intracellular pools of SAM in amoebae and X-bacteria. Graphs for xD amoebae represent the amount of SAM in xD amoebae including that of symbiotic X-bacteria, as measured using a Dowex 50W-X8 resin. The data represent mean ± SE from three separate experiments.

PART IV

DNA ADENINE METHYLATION OF AMOEBA *SAMS1* GENE CAUSED BY X-BACTERIA

* This part is to be submitted for publication as Taeck J. Jeon and Kwang W. Jeon. 2003.

CHAPTER 1

ABSTRACT

The expression of amoeba *sams* genes is switched from *sams1* to *sams2* when amoebae are infected with X-bacteria. Our present data indicate that the *sams1* gene of amoebae is methylated at an internal adenine residue of GATC site in symbiont-bearing xD amoebae whereas no methylation occurs in symbiont-free D amoebae that the modification might be the reason for the inactivation of the *sams1* gene in xD amoebae. Methylation of a cytosine residue in CpG sites is the most common modification in eukaryotes. However, it appears that methylation of cytosine residues is not responsible for the inactivation of the *sams1* gene in xD amoebae. The analysis of DNA shows that adenine residues in X-bacteria *sams* are also methylated, indicating the *Legionella*-like X-bacteria belong to a Dam methylase-positive strain. In addition, it appears that SAM and Met act as negative regulators for the expression of *sams1* whereas the expression of *sams2* is not affected in amoebae. Interestingly, after the removal of X-bacteria from xD amoebae, the *sams1* gene was reactivated and *sams2* was down-regulated. The results are reversed in the expression of two *sams* genes when amoebae are infected with X-bacteria. This is the first report to show that a specific eukaryotic gene is modified by DNA adenine methylation.

CHAPTER 2
INTRODUCTION

S-Adenosylmethionine synthetase (SAMS) is an essential cellular enzyme that catalyzes the formation of S-adenosylmethionine (SAM) from methionine and ATP (Mato et al. 2002; Thomas and Surdin-Kerjan 1997). *Amoeba proteus* contains two *sams* genes, *sams1* and *sams2*. Interestingly, the expression of *sams* genes is switched from *sams1* to *sams2* when amoebae are infected with X-bacteria (Jeon and Jeon in press). However, the mechanism for the switch in the expression of host *sams* genes by endosymbiotic bacteria is not known.

The genomic DNA of most organisms is modified by methylation at the C-5 or N-4 position of cytosine and at the N-6 position of adenine (Low et al. 2001). It has been reported that DNA methylation plays a role in a variety of biological processes such as regulation of gene expression (Oshima et al. 2002), DNA replication, mismatch repair, and in cellular defense against foreign DNA (Bhagwat and Lieb 2002; Low et al. 2001; Karrer and VanNuland 1998). In prokaryotes, adenine and/or cytosine is methylated, depending on the species (Bhagwat and Lieb 2002). Methylation of cytosine residues in CpG sites is a common feature in eukaryotes (Lee 2003; Karrer and VanNuland 1998). So far there has been no report that any endogenous eukaryotic gene is regulated by N6-position methylation of an adenine residue in GATC sequences but some authors suggest that DNA adenine methylation may play a role in the regulation of eukaryotic gene expression (van Steensel and Henikoff 2000; van Blokland et al. 1998).

The xD strain of *Amoeba proteus* arose from the D strain by spontaneous infection of X-bacteria (Jeon and Lorch 1967). In this study, we compared methylation states of the *sams1* genes of D and xD amoebae in the hope of elucidating the mechanism for the switch in the expression of host *sams* genes by endosymbiotic bacteria. We found that some adenine residues of amoeba *sams1* were methylated in xD

amoebae. It appears that DNA adenine methylation of *sams1* may be responsible for the inactivation of *sams1* in xD amoebae.

CHAPTER 3
MATERIALS AND METHODS

Amoebae. The D and xD strains of *A. proteus* were cultured in a modified Chalkley's solution (Jeon and Jeon 1975) in Pyrex baking dishes (35 × 22 × 4 cm). Amoebae were fed daily with axenically cultured and washed *Tetrahymena pyriformis* as food organisms (Goldstein and Ko 1976).

Nuclear Run-On assay. Nuclei were prepared by using a modified method of Choi and Jeon (1989). Amoebae were harvested and homogenized in a buffer consisting of 24-mM n-octanol, 0.03% spermidine, 1.5-mM $MgCl_2$, and 0.5-M sorbitol in 20-mM Tris buffer (pH 7.4). The lysate was filtered through a 45-μM-pore-size nylon screen and the filtrate was layered over 0.5-M sucrose in 20-mM Tris buffer (pH 7.4), containing 0.03% spermidine, 1.5-mM $MgCl_2$, and centrifuged for a total of 5 min at successive accelerations of 70, 250, and 800 *g*. The pellet was resuspended in 20-mM Tris buffer containing 0.5% sorbitol, and laid on top of a 30/20/10% Percoll gradient. Nuclei were collected from the 20/10% interface after centrifugation for 10 min at 1000 *g*, diluted in 5 vol. of 1× run-on reaction buffer, and pelleted by centrifugation for 2 min at 650 *g*.

In-vitro transcription using nuclei was carried out as described by Crowley et al. (1985). The pelleted nuclei (1 x 10^6) were mixed with 400 μl of reaction buffer (40-mM Tris pH 8.0, 10-mM $MgCl_2$, 50-mM KCl, 0.1-mM DTT, 25% glycerol, 400 U of Rnasin, 1-mM each of ATP, GTP, CTP, and 200 μCi of [^{32}P]UTP (ICN, 3000 Ci/mmol) and incubated for 30 min at 30 °C with shaking. The reaction was stopped by the addition of 100 units of DNase I (RNase-free) and 10 μl of 200-mM $CaCl_2$, followed by incubation for 10 min at 37 °C. The samples were subsequently treated with 10 μl of 20 mg/ml proteinase K and 250 μl SET buffer (5% SDS, 50-mM EDTA, 100-mM Tris, pH, 7.5). After additional incubation for 30 min at 37 °C, RNA was isolated by phenol-chloroform and then precipitated by ethanol. Purified ^{32}P-labeled RNA was hybridized to Hybond nylon membrane with immobilized spots containing 5 μg or 10 μg of *sams1*,

myosin, or 18S rRNA. The hybridization was carried out for 2 days at 65°C and the membranes were washed in 0.2x SSC/0.1% SDS for 30 min at 65 °C and in 0.2x SSC/0.1% SDS for 30 min at 37 °C before exposure to X-ray film.

Southern and Western blot analyses. Total DNAs were prepared from D or xD amoebae by DNeasy Tissue kit (Qiagen). For analysis of DNA adenine methylation, 10 μg of DNA was digested with *Dpn*I, *Dpn*II, and *Sau*3AI restriction endonucleases. To test methylation states of cytosine residues in CpG sites, *Bsi*E1 and *Hin*fI were used. All digestions were carried out overnight with a two- to threefold excess of enzyme. Southern blot analysis was performed by a standard procedure. For probing *sams1* and X-bacteria *sams* gene, 0.9-kb fragments (nt -530 to 349) of *sams1* and 0.7-kb fragments (nt -348 to 398) of X-bacteria *sams* (Jeon and Jeon 2003), respectively, amplified by PCR were amplified and labeled with [^{32}P]-dCTP by the Prime-a-Gene labeling system (Promega).

For Western-blot analysis, proteins were extracted from amoebae grown at various concentrations of 5-aza-2′-deoxycytidine (Sigma) for 6 days and immunoblotted with a polyclonal antibody (pAb) against amoeba-SAMS1 as described by Jeon and Jeon (2003).

In-vitro transcription using amoeba nuclear extracts. In-vitro transcription using amoeba nuclear extracts was performed by a modified method of Shapiro et al. (1988). Amoeba nuclei were prepared as described above in nuclear Run-on assay. The pelleted nuclei were resuspended in nuclear resuspension buffer (18-mM HEPES, pH 7.9, 0.675-mM spermidine, 0.135-mM spermine, 0.18-mM EDTA, 2-mM DTT, 22.5% glycerol, 0.42-M NaCl, 0.5-mM PMSF, and 2.5 μg/ml proteases inhibitors) followed by centrifugation for 45 min at 150,000 g. The supernatant was transferred to a new tube and 0.3 g/ml of solid $(NH_4)_2SO_4$ was added. After placing the tube on ice for 20 min, the precipitate was recovered by centrifugation for 20 min at 100,000 g. The protein pellet was resuspended in nuclear dialysis buffer (20-mM HEPES, pH 7.9, 0.1-

M KCl, 0.2-mM EDTA, 2-mM DTT, 20% glycerol) and dialyzed in the same solution. 10 μl of nuclear extracts were used in-vitro transcription containing 10-mM HEPES, pH 7.9, 30-mM KCl, 1-mM each of ATP, GTP, CTP, and UTP.

For DNA templates in the in-vitro transcriptions, the *sams1* gene, from nt -530 to 1212, was amplified and cloned into pGEM easy vector (Promega). Methylated *sams1* was prepared by introducing the plasmid into JM109 and unmethylated *sams1* DNA into INV110 (Invitrogen). As a negative control, 1.1-kb fragments (nt 106 - 1212) of *sams1* was inserted into pGEM easy vector and used. For positive control, pBig-GFPmyo containing actin promoter 15 of dictyostelium was obtained from Dr.James A. Spudich (Moores et al. 1996). The actin promoter 15 was isolated by digestion with *Bam*HI and cloned into pBSKII$^+$ vector.

The RNA was extracted as described in Run-on assay and transcripts of *sams1* and actin were detected by primer-extension experiments described in Jeon and Jeon (2003). To detect the transcripts of *sams1*, antisense oligonucleotides corresponding to nt 33 to 55 relative to the translation initiation site of the *sams1* gene were used in the primer-extension experiments. For detecting the transcripts of actin and *sams1* used as positive and negative controls, a plasmid-specific primer (T7 primer; 5′-TAATACGACTCACTATAGGGCGA-5′) and an oligonucleotide primer complementary to nt 139 to 158 of the *sams1* gene were used, respectively.

PCR-based dam-site methylation analysis. In order to determine dam-site methylation states of 4 dam sites in the 5′-end region of *sams1*, two sets of primers were prepared, one set of primers are located outside 4 dam sites in the 5′-end region and the other located inside 2 dam sites for internal control. Using these primers, PCR were performed after digesting the genomic DNAs of amoebae with *Dpn*I or *Dpn*II. Methylated and unmethylated *sams1* genes were used as a control.

Bisulfite genomic sequencing. Bisulfite genomic sequencing was done as described by Ghoshal et al., (2000). DNA (5 μg) of D and xD amoebae was denatured in 0.3-M

NaOH for 30 min at 37 °C in 10 μl, mixed with 100 μl of 2-M sodium metabisulphite (Sigma) containing 0.5-mM hydroquinone (pH 5.0) and cycled in a thermal cycler for 30 min at 50 °C and for 2 min at 95 °C for 20 cycles. Bisulfite-treated DNA was desalted using a Wizard DNA Clean-up Kit (Promega), eluted in 100 μl of H_2O, and DNA was desulfonated in the presence of 0.3-M NaOH for 30 min at 37 °C. The solution was neutralized by addition of NaOAc (pH 4.5) to 0.2-M (final concentration). The bisulfite-converted DNA was desalted again as before, eluted in 70 μl of H_2O, and an aliquot (0.5 - 1 μl) was used for subsequent PCR amplification.

The promoter region of *sams1* was amplified with two modified primers, SAM1F (5′-CGAATGTAGAAATCATTGGAG-3′) and SAM2R primers (5′-AAATATCCTCTACTAACTAC-3′) corresponding to the nucleotides from nt -530 to -509 and nt 52 to 33 in the *sams1* gene (Jeon and Jeon 2003), respectively, from the bisulfite-treated DNAs of amoebae. To avoid any nonspecific amplification, we amplified the products obtained from the first round of PCR with another set of primers, SAM1F and SAM3R or SAM2R and SAM3F, under the same PCR condition, with the exception that the annealing temperature was maintained at 59 °C. The nucleotides of SAM3R primer (5′-TTTACATCACTACTACAATC-3′) are located at nt -219 to -200 of the *sams1* gene, and SAM3F primer at the same position as SAM3R but in reverse sequences.

Amplified DNA was purified by agarose gel electrophoresis, followed by cleaning the eluted DNA using a Wizard DNA Clean-up kit. The PCR product was directly sequenced using T7 Sequenase Version 2.0 DNA Sequencing Kit (USB) and SAM1F or SAM3F primer.

RT-PCR. Total RNAs of amoebae were extracted by the SV total RNA Isolation System (Promega) after treating amoebae with 1-mM SAM for 3 days or 1-mM Met for 2 days. To remove X-bacteria from xD amoebae, we grew amoebae for 8 days at 27 ˚C. An aliquot (1 μg) of total RNAs was reverse-transcribed with MMLV reverse transcriptase (Promega) and Oligo(dT) primers. The transcripts of amoeba *sams* genes were amplified

with gene specific primers and the products were analyzed in 1% agarose gel. For amplifying *sams1* gene products, we used a forward primer, located at nt –46 to –25, and a reverse primer, located at nt 935 to 916. In the case of *sams2*, a set of primers was used, located at nt 435 to 452 and at nt 732 to 715. Amoeba's myosin primers (Oh and Jeon, 1998) were used as internal controls. The products of RT-PCR were compared with those obtained from amoebae without any treatment.

CHPATER 4
RESULTS

Run-On Assay. The results of Run-on assays using D and xD amoeba nuclei are shown in Fig. 1. The assays were aimed at determining if the inactivation of *sams1* in xD amoebae occurred at the transcriptional or post-transcriptional level. When xD amoeba nuclei were used in the run-on assay, no transcript was detected. However, positive transcripts were detected in the assay using D amoeba nuclei, and the results indicated that the *sams1* gene was inactivated at the transcriptional level in xD amoebae.

Southern blot analysis for DNA adenine methylation in GATC sites of *sams1*. In order to determine why *sams1* was expressed in D amoebae but suppressed in infected xD amoebae, we first checked if this pattern of expression was correlated with dam-site methylation status of *sams1* promoter in D and xD amoebae. For this purpose, we used the methylation-sensitive restriction isoschizomers *Dpn*II and *Sau*3AI (Both enzymes recognize the GATC sequence, but *Dpn*II cannot cleave if the internal A is methylated whereas *Sau*3AI cleaves regardless of the methylation of the recognition sites). Eleven *Dpn*II/*Sau*3AI restriction sites were present in the reported *sams1* gene (Jeon and Jeon 2003). Genomic DNA samples from D and xD amoebae were digested with *Dpn*II or *Sau*3AI, and then Southern blotted using 0.9-kb fragments (nt –530 to 349) of *sams1* as the probe (Fig. 2A). The *sams1* gene in D amoebae was not methylated at GATC sites as indicated by the presence of one fragments of 520 bp in the *Dpn*II-treated samples, consistent with cleavage by *Sau*3AI. In contrast, cleavage of xD-amoeba DNA with *Sau*3AI resulted in the same pattern obtained in D amoebae, but *Dpn*II could not cleave with the same efficiency observed in D amoebae and generated approximately 2 kb of higher molecular-weight bands indicating that adenine residues of some recognition sites of *Dpn*II were methylated (Fig. 2A).

We also analyzed the methylation status of the X-bacteria *sams* gene. Eight restriction sites were present in the *sams* gene of X-bacteria (Jeon and Jeon 2003). The

genomic DNAs of X-bacteria were digested with *Dpn*I, *Dpn*II or *Sau*3AI, and Southern blotted using 0.7-kb fragments of X-bacteria *sams* gene as the probe (Fig. 2B). *Dpn*I recognizes the GATC sequence but cleaves only if the internal A is methylated. When the DNAs were digested with *Dpn*I and *Sau*3AI, a 520-bp fragment was detected, but higher molecular-weight bands in *Dpn*II digests, indicating dam-site methylation in X-bacteria *sams*.

In-vitro transcription using methylated and unmethylated amoeba *sams1* genes. To determine if the dam-site methylation of *sams1* in xD amoebae caused inactivation of the gene, we compared the transcripts of dam-site methylated and unmethylated *sams1* genes by in-vitro transcription experiments using D and xD amoeba nuclear extracts (Fig. 3). In the assay using D-amoeba nuclear extracts, there was no noticeable difference in the transcription of *sams1* between methylated and unmethylated DNAs. Both methylated and unmethylated *sams1* showed a major band of 110 bp corresponding to the size of the fragments transcribed from the transcription start point (nt - 56) of *sams1* (Jeon and Jeon 2003). The DNA without promoter region of the *sams1* gene showed no band and actin DNA used as a positive control showed a band of 160 bp.

In the in-vitro transcription using xD-amoeba nuclear extracts, both methylated and unmethylated *sams1* genes also showed a same size of band detected in the assay using D-amoeba nuclear extracts. This result implies that the region for negative regulation of *sams1* might be present at outside of the gene used in these experiments since the *sams1* gene was completely suppressed in xD amoebae.

Analysis for the methylation states of 4 dam sites in 5′-end region of *sams1*. In order to determine which dam sites of *sams1* were methylated in xD amoebae, we examined the methylation states of four dam sites located in the 5′-end region of the gene (Fig. 4). To test methylation of four dam sites, we amplified 5′-end region of *sams1* by PCR using two primers located outside of the dam sites after digesting

genomic DNAs with *Dpn*I or *Dpn*II. When unmethylated *sams1* was digested with *Dpn*I, a band was detected in the subsequent amplification (Lane 1) whereas no band was present when methylated *sams1* was used (Lane 2). This was because *Dpn*I cleaved dam-site methylated DNAs. When D or xD genomic DNA was used, a same-size band was detected as shown in unmethylated *sams1,* indicating that any of the four dam sites in the 5′-end region of the *sams1* gene was not methylated. In agreement with this result, *Dpn*II digestion showed no amplified band in D and xD genomic DNAs.

Analysis for the CpG methylation in *sams1*. We checked if silencing of the *sams1* gene in xD amoebae was accompanied by changes in CpG methylation of the *sams1* gene. To test CpG methylation states, we performed *Bsi*EI or *Hin*f1 restriction analysis using a full-length amoeba *sams1* as a probe. As seen in Fig. 5, *BsiE*1 treatment of D and xD genomic DNAs showed a same-size band of 1.3 kb. There were four recognition sites for *Hin*f1 in *sams1*, and two bands with sizes of approximately 0.8 and 0.7 kb were detected in *Hin*f1 digests of D and xD genomic DNAs. These results indicated that the recognition sites of *Bsi*E1 and *Hin*f1 in *sams1* were not methylated.

Even though *Bsi*E1 and *Hin*f1 restriction analysis showed no CpG methylation of *sams1* in both D and xD amoebae, it appeared possible that other CpG sites in *sams1* might be methylated in xD amoebae. To test methylation states of other CpG sites in 5′-end region, we performed bisulfite genomic sequencing (Ghoshal et al. 2000). We checked the methylation states of 18 CpG sites out of 21 sites in 5′-end region of *sams1* by bisulfite genomic sequencing and found no methylated CpG site in both D and xD amoebae. Fig. 6 shows only 4 cytosine residues of CpG sites converted to thymines.

In order to determine if methylation of CpG sites played a role in *sams1* silencing in xD amoebae, we tried to modify the methylation status of amoebae by treating them with the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR). For this purpose, amoebae were grown in various concentrations of 5-azadeoxycytidine for 6 days, and SAMS1 expression was assessed by Western blotting using an anti-SAMS1 antibody (Fig. 7). Under these conditions, there was no change and no reactivation of

sams1 expression in D and xD amoebae. Taken together, these results indicated that CpG methylation was not involved in the inactivation of the *sams1* gene in xD amoebae.

Regulation of amoeba *sams* genes. Previously it was shown that the expression of *sams1* was suppressed whereas *sams2* was activated by infection with X-bacteria (Jeon and Jeon 2003). In order to see if the expression of amoeba *sams* genes was reversed to a normal state after removal of X-bacteria, we performed RT-PCR using total RNAs extracted from D and xD amoebae after removing X-bacteria by growing amoebae for 8 days at 27 ˚C (Fig. 8). After growing for 8 days at 27 ˚C, D amoebae showed no change in the expression of *sams1* and *sams2* compared with those grown at normal temperature. In contrast, re-expression of *sams1* was detected in RT-PCR in xD amoebae after the removal of X-bacteria even though no SAMS1 was detected by Western blotting using an anti-SAMS1 antibody (Data not shown). In addition, the *sams2* gene was down-regulated in xD amoebae after removal of X-bacteria (Fig. 8).

In order to gain some insights on the regulation of amoeba *sams* genes by SAM or Met, we measured the expression level of *sams* genes after SAM or Met treatment by RT-PCR (Fig. 8). It was known that SAM or Met acts as a regulator for the regulation of *sams* genes in yeast or mamalians (Corrales et al. 2002; Thomas and Surdin-Kerjan 1997). SAM or Met treatment showed the same changes in the expression of *sams* genes. The expression of *sams1* in D amoebae was down-regulated by SAM or Met treatment but there was no change in the expression of *sams2*. In xD amoebae, no *sams1* transcript was detected by RT-PCR after SAM or Met treatment. Also, there was no difference in the expression of *sams2* in xD amoebae by treatment with SAM or Met compared with control. These data indicated that SAM and Met play a role in the regulation of *sams1* as a negative regulator, but no effect on that of the *sams2* gene.

CHAPTER 5
DISCUSSION

In the present work we examined the methylation status of the *sams1* promoter by using methylation-sensitive restriction enzymes. Our data indicate that the *sams1* gene of amoebae is methylated at an internal adenine residue of GATC site in xD amoebae whereas no methylation occurs in D amoebae. The results suggest that the modification might be the reason for the inactivation of *sams1* in xD amoebae. This appears to be the first case to show that DNA adenine methylation suppresses the expression of a eukaryotic gene. Results of in-vitro transcription experiments using amoeba nuclear extracts suggest that a negative regulatory region is located outside the *sams1* gene. Also, the analysis of methylation states of 4 dam sites in the 5′-end region of *sams1* showed no methylation in both D and xD amoebae.

Additionally, results of Run-on assay using nuclei of D or xD amoebae show that amoeba *sams1* is inactivated at the transcriptional level. It has been reported that the expression of two *sams* genes is switched from *MAT1A* to *MAT2A* in liver-cancer cells (Avila et al. 2000; Huang et al. 1998; Cai et al. 1996) and that the expression of *MAT1A* is suppressed by CpG methylations in the promoter region (Torres et al. 2000). In eukaryotes, the most common modification is methylation of cytosine residues in CpG sites. The switch in the expression of two *sams* genes in liver-cancer cells is similar to that in amoebae caused by infection with X-bacteria (Jeon and Jeon in press). However, DNA analysis using CpG methylation-sensitive restriction enzymes and bisulfite genomic sequencing showed no methylated cytosine residue of the *sams1* gene in both D and xD amoebae. Also, the test for RNA interference by Northern blotting to detect 25 nt specific to RNAi and in-vitro RNAi experiments using D or xD amoeba nuclear extracts showed no positive result (data not shown).

DNA adenine methylation is catalyzed by DNA adenine methylase (Dam) and appears to play a role in the initiation of DNA replication and mismatch repair, phase variation, and gene regulation (Bhagwat and Lieb 2002; Oshima et al. 2002; Low et al.

2001). Dam methylases are found in several strains of Gram-positive and Gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Serratia marcescens*, *Yersinia* spp., *Vibrio cholerae*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Bacillus actinomycetemcomitans*, and *Borelia* sp. (Eberhard et al. 2001; Low et al. 2001). It has been reported that Dam-like methylation is also present in *Legionella* (Lema and Brown 1996) and Dam methylation in *legionellae* may play a role in controlling the piliation and virulence of these organisms (Lema and Brown 1996).

xD Amoebae are composed of two organisms, eukaryotic amoebae and prokaryotic X-bacteria. It is believed that X-bacteria belong to *Legionella* based on the homology of rRNA sequences (K. J. Kim, unpublished data) and of GroEL genes (Ahn et al. 1994). In our study, it was found that adenine residues in X-bacteria *sams* was also methylated. Therefore, it might be postulated that Dam methylase of X-bacteria is transported to their host amoebae and methylate the *sams1* gene. It was reported recently that Dam-deficient *Salmonella typhimurium* are totally avirulent and DNA adenine methylation may control the expression of a large number of genes that participate in the invasion of host cells (Heithoff et al. 1999). However, it is not clear which genes are particularly crucial for the invasion of host cells by *S. typhimurium*. Our data suggest that the expression of host genes also might be affected by Dam methylase of infecting bacteria. It would be useful to know what the effects are on the regulation of host *sams* gene by dam-deficient infecting bacteria and what the relationship is between the suppression of host *sams* gene and the virulence of infecting bacteria.

Another possibility is that Dam methylases of host amoebae might be activated after infection with X-bacteria and then methylate the *sams1* gene. Ciliated protozoa are unusual among eukaryotes in that their genomic DNA shows low levels of DNA adenine methylation, but they have no detectable methylation of cytosine residues (Karrer and VanNuland 1998). Methylated adenine has been reported in several ciliated protozoa including *Tetrahymena* (Karrer and VanNuland 1998), *Paramecium (Cummings et al. 1974)*, *Oxytricha (Rae and Spear 1978)* and *Stylonychia (Ammermann et al.)*, but there is no evidence for any endogenous genes that alter their expression levels in response to DNA adenine methylation. It is desirable to know which organism

is the source of Dam methylase in amoeba/X-bacteria symbiosis in elucidating the mechanism of DNA adenine methylation. Regardless of the source of Dam methylase, our study is the first to show that a specific eukaryotic gene is modified by DNA adenine methylation.

It has been suggested that interactions between regulatory proteins and DNA can be altered by adenine methylation, either through a direct steric effect or as an indirect effect on the DNA structure (Polaczek et al. 1997). Studies with *dam* mutants have shown that Dam methylase regulates the expression of certain genes in *E. coli* including *trpR* (Peterson et al. 1985) Tn*10* transposase(Roberts et al. 1985), *dnaA* (Braun and Wright 1986), and *agn43* (Wallecha et al. 2002). Methylation of adenine in the GATC site(s) within the consensus RNA polymerase binding site inhibits (*trpR* and *Tn*10 transposase) or enhances (*dna*A) transcription, by altering the interaction with the transcription apparatus. However, the exact mechanism by which DNA adenine methylation regulates transcription and the extent of its biological importance remain unclear.

All Dam methylases use SAM as a methyl donor (Low et al. 2001). SAM is known not only as a major methyl donor but also as the precursor for biotin, spermidine, and polyamine (Thomas and Surdin-Kerjan 1997). Additionally, SAM is an intracellular signal to control several essential cellular functions and regulation of *sams* gene itself. It has been reported that the expression of the two *SAM* genes in yeast is regulated differently, *SAM2* being induced by the presence of excess methionine in the growth medium and *SAM1* being repressed under the same conditions (Thomas and Surdin-Kerjan 1997; Thomas and Surdin-Kerjan 1991). In *E. coli*, the *sams* gene is repressed by the addition of methionine to the growth medium (Holloway et al. 1970).

In amoebae, it appears that SAM and Met act as a negative regulator for the expression of *sams1* whereas the expression of *sams2* is not affected. Interestingly, after the removal of X-bacteria from xD amoebae by growing for 8 days at 27 ˚C, the *sams1* gene was reactivated and *sams2* was down-regulated. The results are reversed in the expression of the two *sams* genes when amoebae are infected with X-bacteria. In

Western blot analysis, there was no re-expression of *sams1* even after X-bacteria had been removed from xD amoebae. The discrepancy between protein and RNA levels in the expression of *sams1* after the removal of X-bacteria is due probably to different sensitivities of the experimental procedures.

Further work is in progress to determine which dam sites are critical for the suppression of *sams1* and what kinds of transcription factors are involved in the regulation of the gene. In this study, we show a difference in DNA adenine methylation of the *sams1* gene between D and xD amoebae. In the previous paper, it was shown that there was no difference in the *sams1* gene between D and xD amoebae. Additionally, in our study, we show that RNAi or CpG methylation is not responsible for the suppression of *sams1* in xD amoebae. Therefore, it appears that the expression of *sams1* in xD amoebae is suppressed by a novel mechanism, DNA adenine methylation, in the eukaryotic gene regulation. The mechanism for the suppression of amoeba *sams1* by X-bacteria would be a good example for the alteration of host gene expression in the interactions between hosts and infective agents such *as Mycobacterium, Legionella, Toxoplasma, Salmonella,* and others.

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APPENDIX

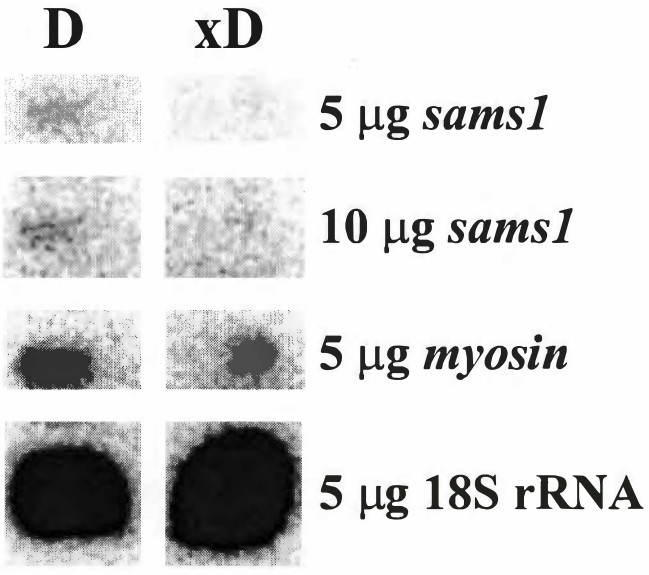


Fig. 1. Run on Assay. The nuclei (1 x 10^6) of amoebae were prepared and used in-vitro transcription. Purified ^{32}P-labeled RNA was hybridized to Hybond nylon membrane with immobilized spots containing 5 μg and 10 μg of *sams1*, *myosin*, and 18S rRNA of amoebae.

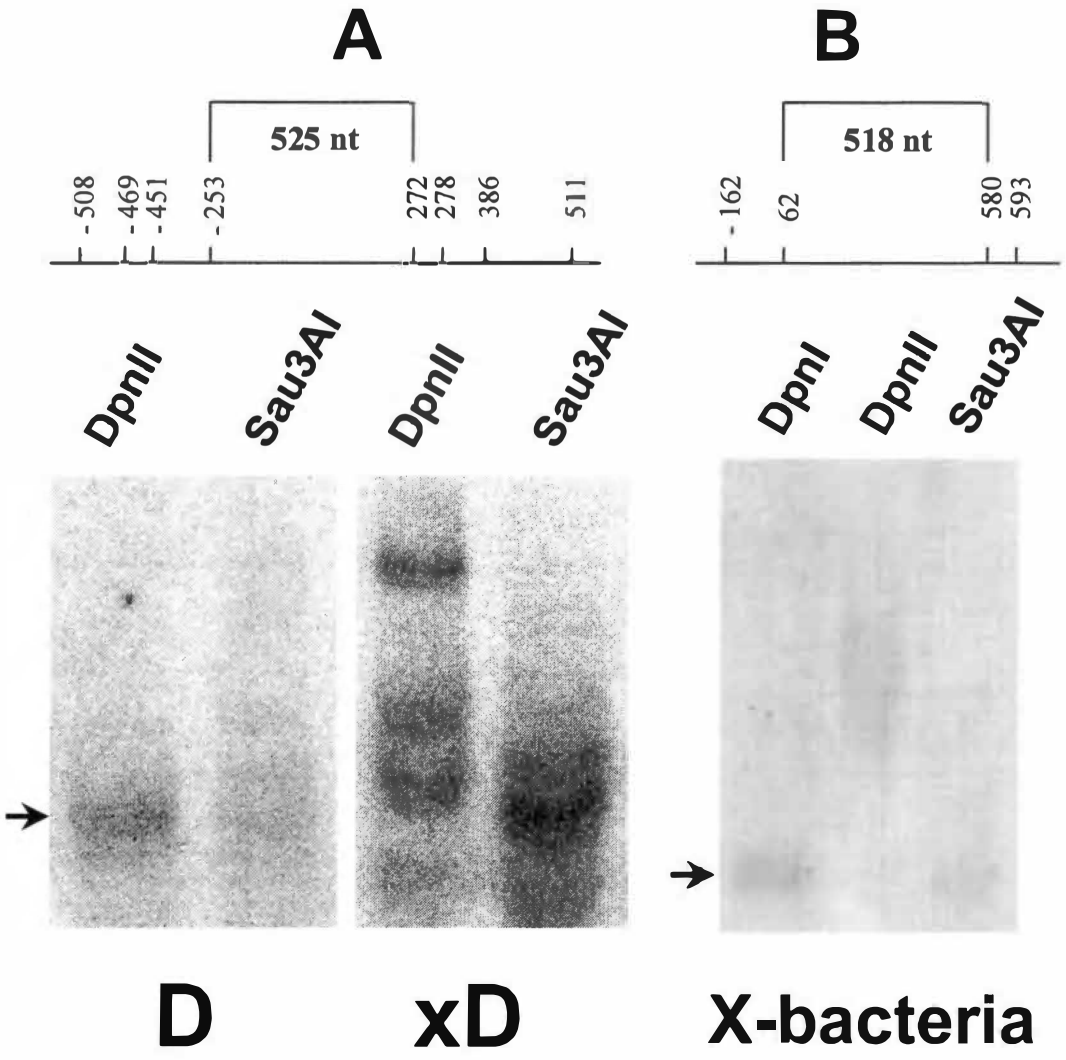


Fig. 2. Southern blot analysis for DNA adenine methylation. The DNAs (10 μg) of D and xD amoebae (**A**) or X-bacteria (**B**) were digested with *Dpn*I, *Dpn*II, and *Sau*3AI restriction endonucleases. For probing *sams1* and X-bacteria *sams* gene, 0.9-kb fragments (nt −530 to 349) of *sams1* and 0.7-kb fragments (nt −348 to 398) of X-bacteria *sams* (Jeon and Jeon 2003) were used, respectively. Restriction map of 5′-end promoter region of amoeba *sams1* and X-bacteria *sams* shows recognition sites of the restriction enzymes and the sizes of the fragments detected in blots.

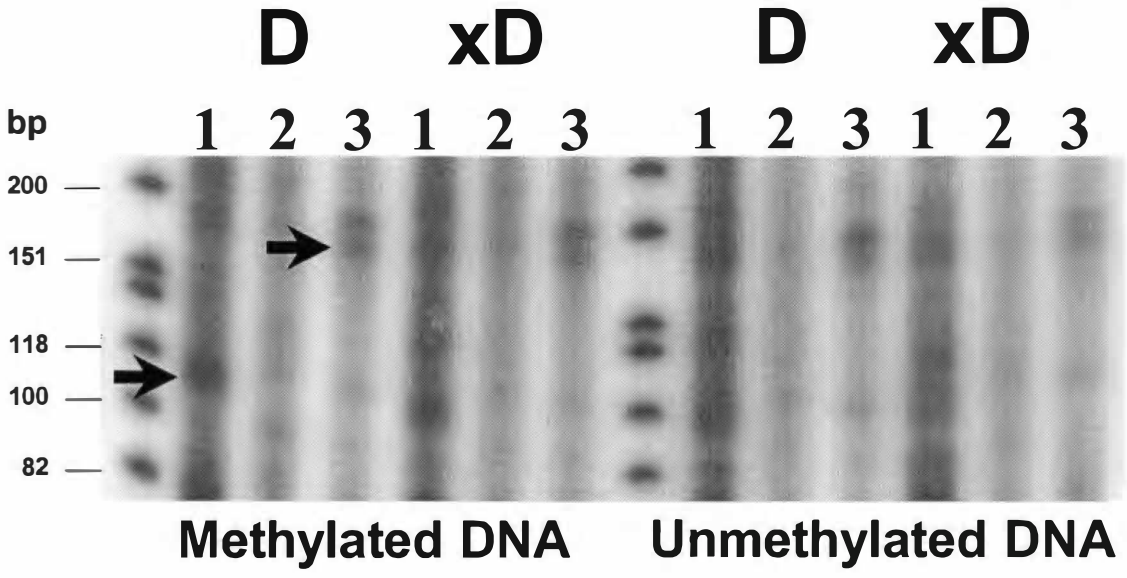


Fig. 3. In-vitro transcription using amoeba nuclear extracts. Methylated and unmethylated amoeba *sams1* genes (Lane 1), located from nt –530 to 1212, were prepared and used in vitro transcription using amoeba nuclear extracts as DNA templates. As a size marker, ϕx174 DNA/Hif1 marker (Promega) was end-labeled with [γ-^{32}P]ATP using a T4 polynucleotide kinase. Amoeba *sams1* gene (nt 106 to 1212) was used as a negative control (Lane 2) and as a positive control (Lane 3), actin promoter 15 of dictyostelium was used. Arrows indicate the transcripts of *sams1* and *actin*, respectively.

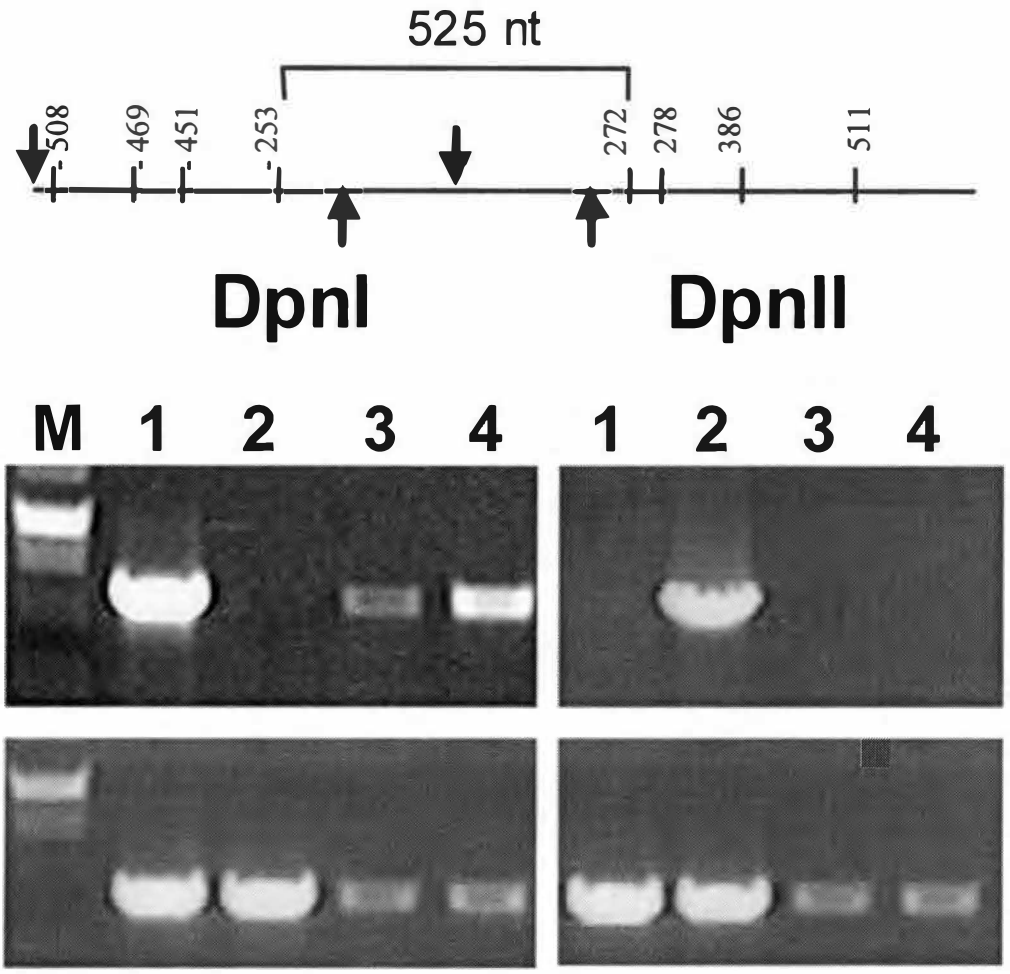


Fig. 4. Analysis for the methylation states of 4 dam sites in 5′-end region of *sams1*. Restriction map shows the recognition sites of *Dpn* restriction enzymes. Arrows show two sets of primers, one set of primers located outside 4 dam sites in the 5′-end region and the other located inside 2 dam sites. Using these primers, PCR were performed after digesting D (Lane 3) and xD amoeba (Lane 4) genomic DNAs with *Dpn*I or *Dpn*II. Unmethylated (Lane 1) and methylated (Lane 2) *sams1* genes were used as a control.

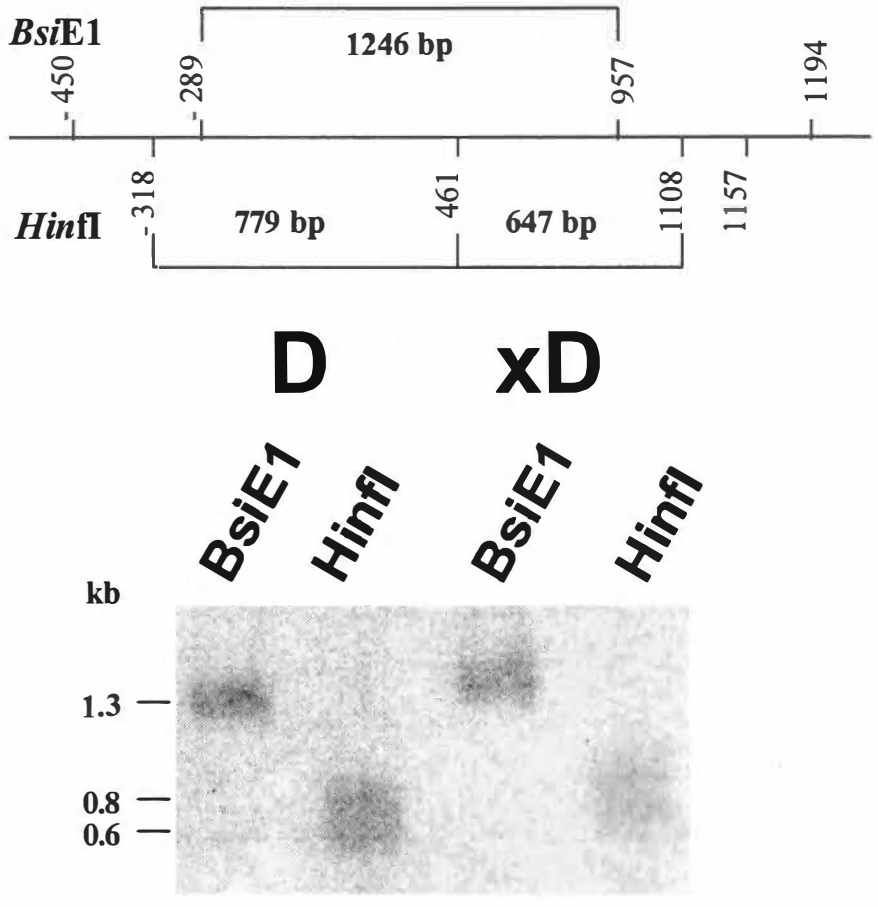
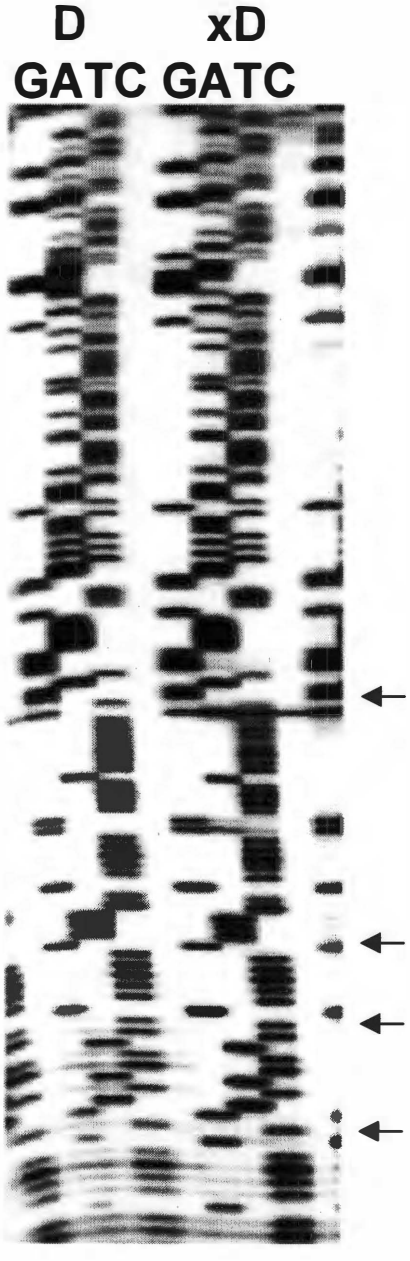


Fig. 5. Southern blot analysis for the CpG methylation in *sams1*.

The recognition sites of *Bsi*E1 and *Hin*fI are shown in a restriction map. D and xD amoeba genomic DNAs were digested with *Bsi*E1 or *Hin*fI and the whole *sams* gene (Jeon and Jeon 2003) was used for detection of the restriction fragments.

Fig. 6. Bisulfite genomic sequening for the promoter region of *sams1*. The bisulfite-treated D and xD amoeba *sams* genes were amplified with SAM1F and SAM2R primers and then SAM3F and SAM2R primers. The products amplified in the second round of PCR were sequenced using T7 Sequenase Version 2.0 DNA Sequencing Kit (USB) and SAM3F primer. Arrows indicate the cytosine residues of CpG sites converted to thymines in both D and xD amoebae.


D
GATC
xD
GATC

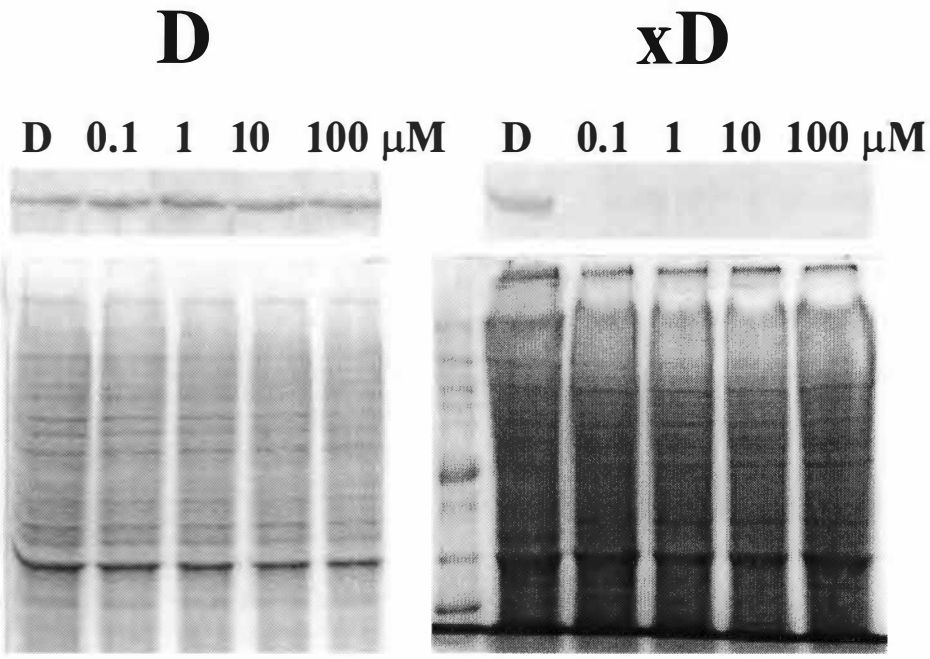


Fig. 7. Western blot analysis for the expression of *sams1* after treating amoebae with 5-azacytidine. D and xD amoebae were treated with 5-azacytidine at various concentrations for 6 days. The expression of sams1 was determined by immunoblotting with a pAb against amoeba-SAMS1.

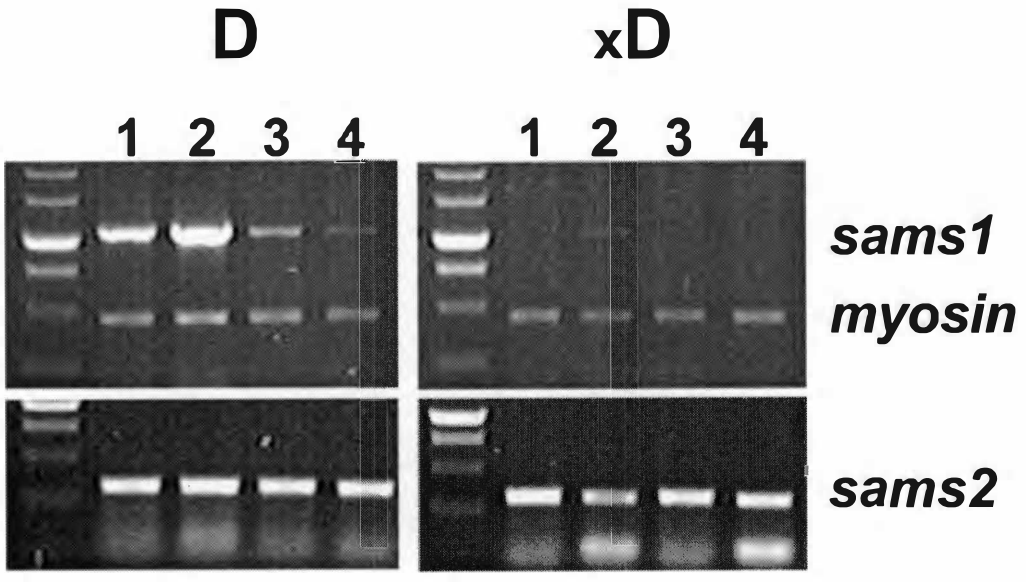


Fig. 8. RT-PCR analysis for *sams* genes. Total RNAs of amoebae were extracted after growing amoebae for 8 days at 27 ˚C (Lane 2) and treating with 1-mM SAM for 3 days (Lane 3) and 1-mM Met for 2 days (Lane 4), respectively. Amoebae without any treatment were used as controls (Lane 1). The cDNAs were amplified with *sams1*-, *sams2*-, or *mysosin*-specific primers and visualized in 1% agarose gel.

PART V

CONCLUSIONS AND FUTURE PERSPECTIVES

This study was focused on the question why the hosts become dependent on symbionts. It has been previously reported that symbiont-bearing xD amoebae do not transcribe the *sams* gene and no longer produce their own S-adenosylmethione synthetase (SAMS) as a result of harboring X-bacteria. Meanwhile, xD amoebae still show about half the level of SAMS activity found in symbiont-free D amoebae, in spite of the fact that they do not produce their own SAMS. The *sams* gene is essential for the survival of the cell. The central hypothesis in this study was as follows: X-bacteria suppress the expression of amoeba's *sams* and in turn provide the enzyme of SAMS for their hosts, forcing host amoebae to become dependent on symbionts themselves. The specific Aim 1 was to investigate the compensation of X-bacteria for the deficiency of SAMS in the host amoebae and the relationship between a host's dependence on its symbionts for survival and the compensation of SAMS or SAM by symbionts. The specific Aim 2 was to study the mechanism for the inactivation of amoeba *sams* gene by infection with X-bacteria.

In this study, I first completed an amoeba-*sams* gene, which was missing 5′-end region in the previous report, and extended 5′-end genomic sequences. The open reading frame of the amoeba's *sams* gene had 1,281 nucleotides, encoding SAMS of 426 amino acids with a mass of 48 kDa and pI of 6.5. The *sams* gene of X-bacteria was also cloned. The open reading frame of X-bacteria *sams* was 1,146 nucleotides long, encoding SAMS of 381 amino acids with a mass of 41 kDa and pI of 6.0. The X-bacteria SAMS had 45% sequence identity with that of *A. proteus*. When symbiont-free D amoebae were infected with symbionts (X-bacteria), the amount of amoeba SAMS and transcripts of *sams* decreased to a negligible level within four weeks after infection, but about 50% of the SAMS activity.

While studying the role of SAMS in amoeba-bacteria symbiosis, I found a second *sams* (*sams2*) gene of amoebae, encoding 390 amino acids. Localizing X-bacteria SAMS in xD amoebae and measuring SAMS activities or SAM amounts in D and xD amoebae showed that the half SAMS activity found in xD amoebae came from SAMS2 of amoebae not from their endosymbionts. Interestingly, the expression of amoeba *sams*

genes was switched from *sams 1* to *sams2* by infection with X-bacteria, raising a possibility that the switch in the expression of host *sams* genes by bacteria plays a role in the development of symbiosis and the host-pathogen interactions. This is the first report showing the switch in the expression of host *sams* genes by infecting bacteria. These results are similar to the switch in the gene expression from *MAT1A* to *MAT2A*, which is encoding SAMS, in human liver cancer and from 12 to 24 h after partial hepatectomy in the rat. Further work is in progress to determine the consequences of the gene switching in amoeba/X-bacteria symbiosis.

As a mechanism for the inactivation of amoeba *sams1* gene by infection with X-bacteria, I have tested several possibilities. First, Run-on assay using nuclei of D or xD amoebae showed that the expression of *sams1* was inactivated at the transcriptional level by infection with X-bacteria. Complete nucleotide sequences of *sams1* genes of D and xD amoebae showed that there was no difference between the two. Long-established xD amoebae contained an intact *sams* and thus the loss of xD amoeba's SAMS was not due to the loss of the gene itself. Neither RNAi nor CpG methylation was the reason for the inactivation of the *sams1* gene by infection with X-bacteria.

Interestingly, while checking the methylation status of the *sams1* promoter by DNA analysis using methylation-sensitive restriction enzymes, I found that the *sams1* gene of amoebae was methylated at an internal adenine residue of GATC site in xD amoebae whereas no methylation occurred in D amoebae. The results suggest that the modification might be the reason for the inactivation of *sams1* in xD amoebae. This appears to be the first case to show that DNA adenine methylation suppresses the expression of a eukaryotic gene. The results of in-vitro transcription experiments using amoeba nuclear extracts suggested that a negative regulatory region was located outside the *sams1* gene. Also, the analysis for methylation states of 4 dam sites in the 5′-end region of *sams1* showed no methylation in both D and xD amoebae.

DNA adenine methylase (Dam) catalyzes DNA Adenine methylation playing a role in the initiation of DNA replication and mismatch repair, phase variation, and

gene regulation. Dam methylases are found in several strains of Gram-positive and Gram-negative bacteria including *Legionella*. Based on the homology of rRNA sequences and of GroEL genes, it is believed that X-bacteria belong to *Legionella*. In my study, it was found that adenine residues in X-bacteria *sams* were also methylated. Therefore, it might be postulated that Dam methylase of X-bacteria is transported to their host amoebae and methylate the *sams1* gene. It was reported recently that Dam-deficient *Salmonella typhimurium* are totally avirulent and DNA adenine methylation may control the expression of a large number of genes that participate in the invasion of host cells. However, it is not clear which genes are particularly crucial for the invasion of host cells by *S. typhimurium*. My data suggest that the expression of host genes also might be affected by Dam methylase of infecting bacteria. It would be useful to know what the effects are on the regulation of host *sams* gene by dam-deficient infecting bacteria and what the relationship is between the suppression of host *sams* gene and the virulence of infecting bacteria.

Another possibility is that Dam methylases of host amoebae might be activated after infection with X-bacteria and then methylate the *sams1* gene. Methylated adenine has been also reported in several ciliated protozoa even though there is no evidence for any endogenous genes that alter their expression levels in response to DNA adenine methylation. It is desirable to know which organism is the source of Dam methylase in amoeba/X-bacteria symbiosis in elucidating the mechanism of DNA adenine methylation. Regardless of the source of Dam methylase, my results are the first to show that a specific eukaryotic gene is modified by DNA adenine methylation.

Further work is in progress to determine which dam-sites are critical for the suppression of *sams1* and what kinds of transcription factors are involved in the regulation of the gene. The switch in gene expression in amoebae and the mechanism for the suppression of amoeba *sams1* by X-bacteria would be a good example for the alteration of host gene expression in the interactions between hosts

and infective agents such *as Mycobacterium, Legionella, Toxoplasma, Salmonella,* and others.

VITA

Taeck-Joong Jeon was born in Kyungbuk Province, Korea on January 1, 1968. He attended Bugoo Elementary School, Bugoo Junior High School, and graduated from Jook-Byun High School in February of 1986. In March of 1987, he entered Korea National University of Education and received the degree of Bachelor in Biology in February of 1994 after serving in the Korean Army for three years. He continued his graduate study and in February of 1997, received the degree of Master of Education in Biology Education from the same university. He enrolled in the Graduate School at the University of Tennessee, Knoxville in August of 1998 and graduated with a Doctor of Philosophy in Biochemistry and Cellular and Molecular Biology in August of 2003. He was married to Hyona Baek in January of 1999 and they have a son, Jonghee Jeon.